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THYROID FUNCTION AS DISCLOSED BY NEWER METHODS OF STUDY

BY

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THYROID FUNCTION AS DISCLOSED BY NEWER METHODS OF STUDY*

Consulting Editor: J. H. MEANS

CONTENTS

	PAGE
Introduction to the Conference. By J. H. MEANS	281
Phylogeny of the Thyroid: Descriptive and Experimental. By E. D. GOLDSMITH	283
Cytological and Cytochemical Bases of Thyroid Function. By E. DE ROBERTIS	317
The Chemical Cytology of the Thyroid Gland. By EDWARD W. DEMPSEY	336
The Metabolic Circuit of the Thyroid Hormone. By WILLIAM T. SALTER	358
Studies on the Formation of Organically-Bound Iodine Compounds in the Thyroid Gland and Their Appearance in Plasma as Shown by the Use of Radioactive Iodine. By I. L. CHAIKOFF AND ALVIN TAUROG	377
Iodine Absorption and Utilization under the Influence of Certain Goitrogens. By D. A. MCGINTY	403
Mechanisms of Action of Various Antithyroid Compounds. By E. B. ASTWOOD	419
Studies on the Metabolism of Thyroxine in the Body. By C. P. LEBLOND	444
The Formation of Thyroxine in Iodinated Proteins. By E. P. REINEKE	450
The Biochemistry of the Thyrotropic Hormone. By A. ALBERT	466
Physiological Reactions of the Thyroid-Stimulating Hormone. By RULON W. RAWSON	491

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* This series of papers is the result of a Conference on Thyroid Function as Disclosed by Newer Methods of Study, held by the Section of Biology of The New York Academy of Sciences on January 24 and 25, 1947.

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INTRODUCTION TO THE CONFERENCE

By J. H. MEANS

Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts

WHEN, nearly a year ago, I received an invitation from Dr. Ross F. Nigrelli to prepare a program for a conference on *Thyroid Action and Anti-Thyroid Drugs*, I accepted with enthusiasm. However, I suggested that the title of the conference be changed to *Thyroid Function as Disclosed by Newer Methods of Study*. It seemed to me that it would be more profitable to discuss several new approaches to thyroid function than limit the discussion to anti-thyroid drugs alone.

Previous to the Conference, I received a telegram from Jane Stafford of Science Service asking for the gist of my introductory remarks, and hoping that I would include a statement on present or future practical aspects of current thyroid research. I wired back that the purpose of the Conference is to explore fundamental physiology of the thyroid gland, and not to concern itself with practical applications. Even to one like myself, who by training can approach thyroid problems only on the clinical level, as the years go by their fundamental aspects become increasingly intriguing. The make-up of the program gives assurance that the objective will be reached. Among the contributors we find anatomists, biochemists, physiologists, pharmacologists, and clinical endocrinologists. All are distinguished scientists who have explored, by various routes, the deep secrets of the thyroid. Unquestionably, the work of each is familiar to all the others, but it is my expectation that, by coming together in this way, the fecundity of aggregation, as Walter Cannon loved to call it, may become operative and that these scientists, by their deliberations, may advance our subject further than if they had not come together here. There are present, too, not listed in the program, others who are equally qualified to contribute to the Conference. I hope that they will take part in the discussion.

It is quite fitting to have a meeting of this kind just now because, in recent years, new methods of investigation have made possible the accumulation of much new knowledge. Among such methods, or approaches, may be mentioned tracer studies by means of radioactive iodine; modification of thyroid function, at will, by means of anti-thyroid or goitrogenic drugs; the techniques of enzyme chemistry; histo- and cyto-chemical methods; tissue culture techniques; microdissection; hormone assays (bio- and chemical); and doubtless others.

What questions are we seeking to answer by such methods? It seems to me they have to do with such matters as the mechanism of thyroid hormone synthesis; the storage, delivery, and transport of thyroid hormone; the agencies through which these functions are controlled; how the thyroid hormone reaches and impinges upon its end-organs, the cells of the body; how it affects the end-organs; and what becomes of the hormone after it has exerted its action.

Then, coming to the pituitary hook-up, we want to explore these same functions as they relate to the thyrotropic hormone. For instance—What also is the rate and manner of secretion of this hormone? How is it controlled? How does this hormone act on its end-organ—the thyroid cell? and, How is the whole pituitary thyroid axis controlled from without; that is to say, through the nervous system, or through the influence of other of the endocrine glands?

Some of these questions will be answered by our contributors, others will be debated—perhaps clarified—though not answered.

There is one approach that I have not yet mentioned. That is the phylogenetic. It is one of which I know but little, though it rather fascinates me. I am curious about how we acquired these endocrine glands which we are discussing, and I wonder if an understanding of their evolution may not contribute to our knowledge of their present functioning. Was the thyroid once a gland of external secretion? If so, how did it get changed to one of internal secretion? Why is it that, whereas most of the endocrine glands of the present-day vertebrate appear to be under the control of the pituitary, the parathyroids and (as I learned from Dr. Roy Greep the other day) the glomerulosa portion of the adrenal cortex seem not to be under pituitary control? What signifies it, moreover, that while the thyroid is chiefly under pituitary control, it also seems, to some degree, directly to control itself? Do those endocrine activities which are not controlled by the pituitary represent older functions than those that are under pituitary control? I have been curious for years about the endocrinology of invertebrates, but, alas, never had the training to explore it. There seems to be rather scant information on the subject. The first I ever got was from the 1945 Dunham Lecturer at Harvard, Dr. Vincent B. Wigglesworth, Director of the Unit of Insect Physiology of the London School of Tropical Medicine. In some very beautiful experiments on a certain tick, he showed very clearly that hormones of its own play a role in both the growth and maturation of this insect.

I think a great deal can be done in the field of comparative endocrinology. If I had the money, I would found a department of comparative endocrinology, but I have not. However, because of these interests I was delighted to be able to induce Dr. E. D. Goldsmith of New York University to open our program with a talk on the phylogeny of the thyroid gland.

PHYLOGENY OF THE THYROID: DESCRIPTIVE AND EXPERIMENTAL*

By E. D. GOLDSMITH

Department of Histology, College of Dentistry, New York University, New York, N. Y.

> IN NATURE'S INFINITE BOOK OF SECRECY
> A LITTLE I CAN READ.
>
> *Shakespeare*

IN the preface to his excellent volume, *Our Face from Fish to Man*, Dr. William King Gregory[1] states that "this book does not pretend to tell how to improve one's face but only how and why one has one." In the same vein, the following is a presentation of a few facts, some descriptive and others pertaining to the experimental, as to the origin and relationship of the thyroid gland in the animal series. The study of function, mechanism, and treatment of glands running wild is left to the authors who follow.

Descriptive

In the human, clasping the upper end of the trachea, lies the thyroid gland, first accurately described and named by Thomas Wharton[2] in 1659. It arises early in fetal life, toward the end of the fourth week, by a downgrowth of epithelium from the ventral wall of the pharynx between the first and second pharyngeal arches (FIGURE 1a). With the forward growth of the pharynx, the gland primordium appears to grow caudally, dividing into a right and left lobe, remaining attached to the pharynx by a stalk or neck, the thyroglossal duct. This structure temporarily connects the young thyroid with the posterior border of the developing tongue. During the fifth week, the thyroglossal duct atrophies, but its site of origin remains as the *foramen caecum* in post-uterine life. Segments of the thyroglossal duct which do not become obliterated may give rise to thyroglossal cysts (FIGURE 1b).

Prior to the development of the various branches of experimental biology, much thought and effort were expended in the study of the origin of the vertebrates and interrelationships of the invertebrates. Examination of the phylogenetic tree (FIGURE 2) reveals the animal relationships as derived from the descriptive evidence presented by the embryologist and morphologist.

As we descend the tree, we find that development of the thyroid gland is similar, with minor exceptions, in the various mammals (man,[3,4] pig,[5,6] calf,[7] horse,[8] dog,[9] rat,[10,11,12] guinea pig,[13] mole,[14,15] bat,[16] *Tar-*

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* The original research here cited of the author and his associates was aided by grants from the Commonwealth Fund, Josiah Macy, Jr., Foundation, and the Elizabeth Thompson Science Fund. The colored plates were generously supplied by Dr. John R. Mote, Medical Director, The Armour Laboratories, Armour and Company.

sius[17]); in birds,[18-22] in reptiles,[23, 24] and Amphibia.[25-29] The basic similarity is exemplified in a urodele, *Necturus*, where the thyroid arises as a single median diverticulum from the pharynx, and grows caudally, splitting into a right and left lobe and leaving an anterior median mass.[29-32] In the adult *Necturus*, Charipper[33] has described the thyroid as consisting of three masses, the more anterior median one often connected to the lateral masses by a tract of follicles reminiscent of the persistent thyroglossal duct.

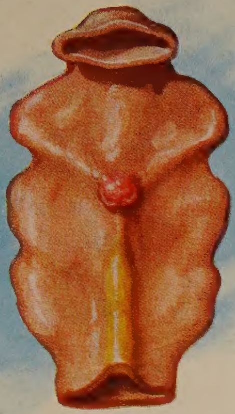
In *Squalus acanthias*, an elasmobranch, the thyroid arises as a solid epithelial bud in the region ventral and caudal to the ventral borders of the first and second gill pouches in embryos approximately 4 mm. in length, with follicles developing as they do in the human.[34, 35] The gland itself is said to be encapsulated. As for the thyroglossal duct, it was reported in *Squalus acanthias* by W. Müller[36] and in a primitive selachian, *Chlamydoselachus*, by Goodey,[37] but was not observed in *Squalus* by Norris.[35]

In a ganoid, *Amia*,[38] the thyroid primordium arises as a solid median outgrowth from the pharynx, becoming detached early in development (FIGURE 3), leaves no thyroglossal duct, and migrates to a position between the bases of the third visceral pouches ventral to the aorta. In the adult fish, the gland consists of a group of follicles scattered in the ventral pharyngeal region around the aorta.[38] Hill associated the thyroid primordium with the region of the first and second visceral pouches, corresponding thus to the location of the thyroid primordium in the teleosts studied by Maurer.[39]

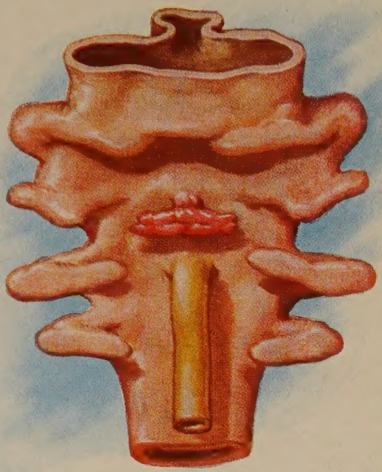
The thyroid of the teleost was first reported in 1844 by Simon,[40] who erroneously believed it to be absent in the salmon and trout. The teleost thyroid has been described[39, 41-45] as constituted of numerous units, the follicles scattered over a wide area. In its development, the trout thyroid arises as a vesicular evagination from the floor of the pharynx in the region between the first and second gill pouches.[39] From his comprehensive survey of the thyroid gland in the teleost, Gudernatsch[41] arrived at the same conclusion. More recently, Hoar,[45] in his studies of the Atlantic salmon, found that the thyroid anlage develops as a solid knoblike structure from the mid-ventral floor of the pharynx at the posterior margin of the hyomandibular junction. These data are in agreement with those reported for the dipnoan, *Ceratodus*.[46] With regard to the capsule present in the elasmobranchs and in all higher forms, it should be noted that there is a very great range in the degree of compactness of the thyroid tissue in the teleosts.[41, 42] More specifically, in the salmon, *Salmo salar*, in very large individuals, the acini may be crowded together to form a compact and nodular gland.[45]

(*See opposite page*)
FIGURE 1a. The development of the thyroid gland. (From *The Thyroid Gland*, The Armour Laboratories, 1943. Drawings by Dr. Frank Netter.)

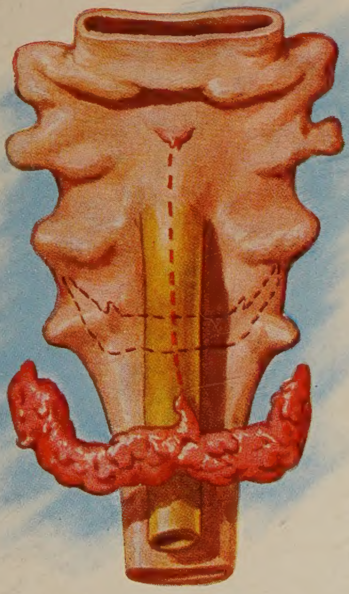
Development of the Thyroid Gland
(SCHEMATIC)



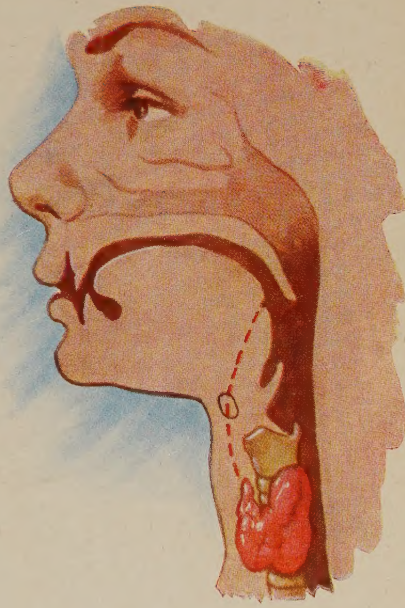
3 mm. embryo—fourth week. Thyroid anlage developing as a bud from ventral surface of primitive foregut. Primitive trachea "yellow" budding off from pharynx.



10 mm. embryo — sixth week. Thyroid enlarging, trachea has developed into a tube.



15 mm. embryo — seventh week. Thyroid has migrated down in front of trachea. Thyroglossal tract obliterated. Black broken line indicates hyoid bone.

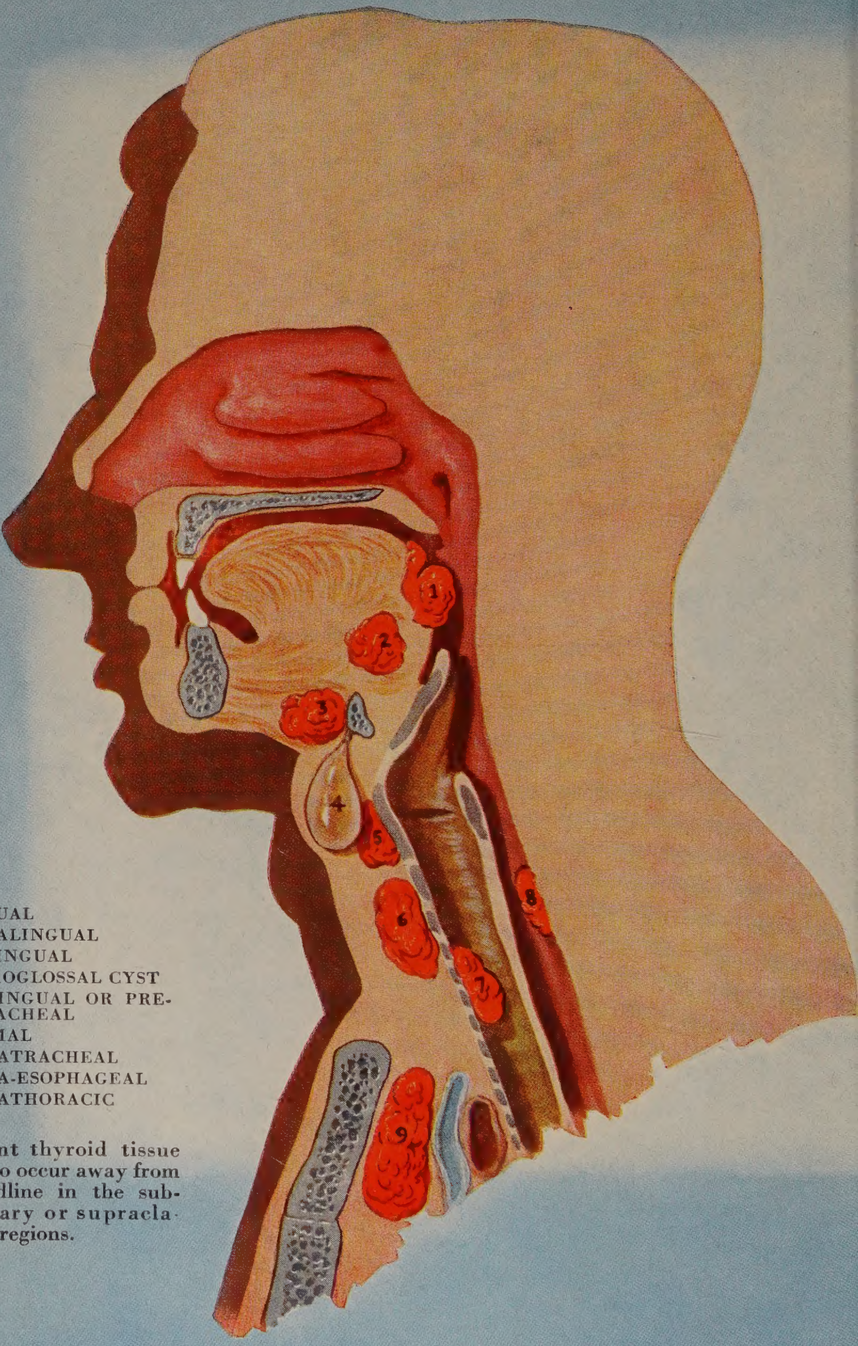


Sagittal section to show post-uterine condition.

F. Netter M.D.

Courtesy of THE ARMOUR LABORATORIES

Sites of Aberrant Thyroid Tissue



1. LINGUAL
2. INTRALINGUAL
3. SUBLINGUAL
4. THYROGLOSSAL CYST
5. PRELINGUAL OR PRETRACHEAL
6. NORMAL
7. INTRATRACHEAL
8. INTRA-ESOPHAGEAL
9. INTRATHORACIC

Aberrant thyroid tissue may also occur away from the midline in the submaxillary or supraclavicular regions.

F. Netter, M.D.

Courtesy of THE ARMOUR LABORATORIES

Cyclostomes and Protochordates. Upon examination of the adult cyclostomes, one finds a thyroid gland consisting of a small number of isolated follicles.[47–53] This is the most primitive thyroid, and a study of the life history of the cyclostomes reveals several interesting facts. Prior to its becoming an adult, the cyclostome passes through a larval stage. To the larva of the cyclostome, *Petromyzon*, the name *Ammocoetes* was given in the mistaken belief that it was an adult member of a distinct genus. The general appearance of this larva is somewhat like that of another organism lower in the phylogenetic series, *Amphioxus*. Both burrow on the bottoms of streams and feed on microscopic organisms. In *Amphioxus* (FIGURE 4) and in a related protochordate, the ascidian or tunicate (FIGURE 5), the mid-ventral line of the pharynx is marked by conspicuous grooves, the hypobranchial groove or endostyle. It is of pharyngeal origin (FIGURE 6), is composed of cuneiform cells, and is believed to secrete mucus. The food is driven into the pharynx by cilia, is entangled in a mass of mucus secreted by the endostyle,* and is then passed by cilia on the gill bars to the esophagus.

In the *Ammocoetes* larva, below the first to fifth gill arches, there is what appears to be a similar groove which arises as a trough in the floor of the pharynx. This structure, too, has been called "endostyle." Arising from the floor of the pharynx, it pinches off, remaining connected during larval life by a duct opening between the third and fourth gill pouches. Alcock[54] studied the digestive effects of a number of *Ammocoetes* tissues on fibrin, but could find no proteolytic ferment in the endostyle. Hyman[55] is of the opinion that this organ does not resemble the endostyle of protochordates and appears not to function in feeding. As the result of a careful survey, Leach[56] concludes that "no comparable anlage of definitive thyroid follicles exists in the endostyle of the protochordates."

Call this structure what you will, endostyle or hypobranchial groove, we do know that it persists for 3 or 4 years and then, at metamorphosis, first described by A. Müller[57] in 1856, it undergoes involution with but a small portion remaining. During this period, it undergoes a series of profound changes which affect the various cellular components. W. Müller[58] compared the endostyle of the tunicates, *Amphioxus* and *Ammocoetes*, suggesting that the thyroid gland of the adult chordate was homologous to it. This point of view was supported by Anton Schneider.[59] As a result of a careful study of the lamprey, *Ammocoetes branchialis*, Marine[60, 61] reported a progressive decrease in the size of the endostyle chambers, with changes in epithelial cells resulting in the formation of up to four ductless follicles in each half of the endostyle from type IV

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* Up to the time of Galen, it was believed that the thyroid gland aided in the lubrication of the pharynx and trachea.

(See opposite page)

FIGURE 1b. Sites of aberrant thyroid tissue. (From *The Thyroid Gland*, The Armour Laboratories, 1943. Drawings by Dr. Frank Netter.)

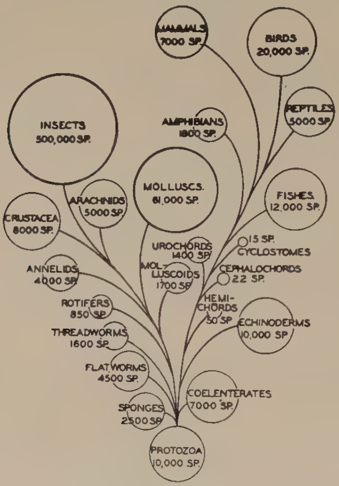

FIGURE 2. Phylogenetic tree. (From NEAL & RAND, *Comparative Anatomy*, Blakiston Co.)

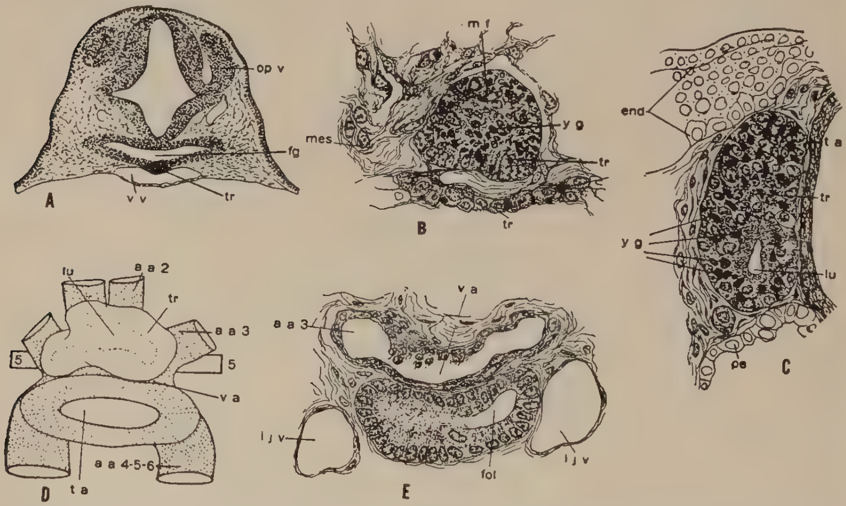

FIGURE 3. (After B. H. HILL.[38]) *A*. Frontal section through thyroid primordium of *Amia* 1 day old. *B*. Transverse section through thyroid of *Amia* 1 day old. *C*. Sagittal section through thyroid of *Amia* 2 days old. *D*. Graphic reconstruction of thyroid region of *Amia* of 8 mm., ventral aspect. Dotted lines show the outline of the lumen of the thyroid. *E*. Transverse section through thyroid of fig. *D* at line *5–5*.

Abbreviations: *aa 1–6*, aortic arches 1–6; *end*, endoderm; *fg*, foregut; *fol*, thyroid follicle; *ijv*, inferior jugular vein; *lu*, lumen; *mes*, mesenchyme; *mf*, mitotic figure; *opv*, optic vesicle; *pe*, pericardium; *ta*, *truncus arteriosus*; *va*, ventral aorta; *vv*, ventral vessel; *yg*, yolk granule; *tr*, thyroid *primordium*.

epithelium. New follicles were said to arise by budding from the primary ones (see FIGURES 7 and 8).

In more recent papers, Leach[56, 62] has described the structure of the

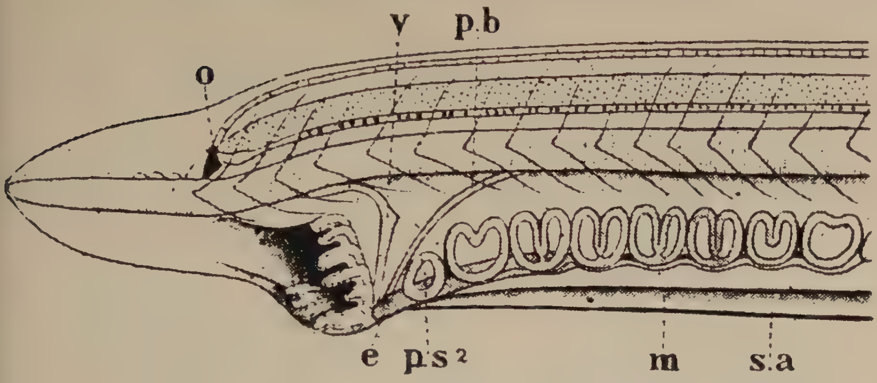

FIGURE 4. *Amphioxus* larva toward the close of metamorphosis. (From WILLEY, 1894.) *e.*, endostyle; *p.b.*, peripharyngeal band; *p.s.2*, secondary primary slit.

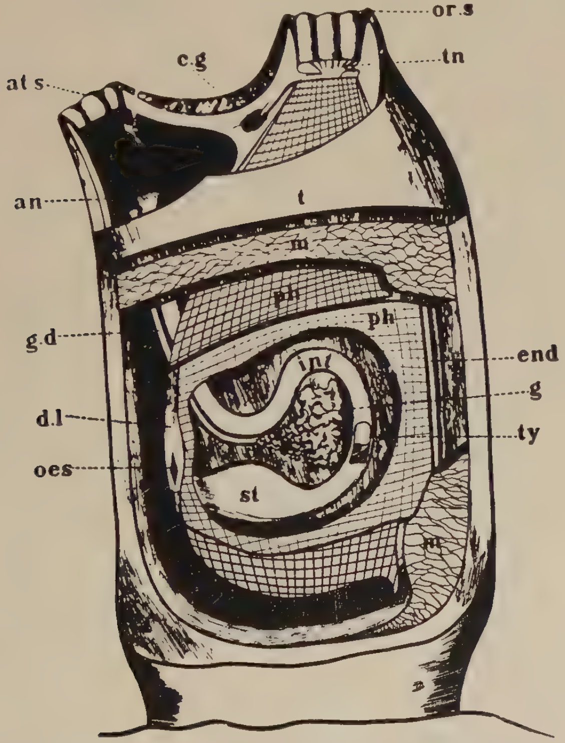

FIGURE 5. Diagram of a dissection of *Ascidia*; from the right side. (From WILLEY, 1894.) The peribranchial cavity is indicated by the black shading. *an.*, anus; *at.s.*, atrial siphon; *c.g.*, cerebral ganglion, beneath which is the subneural gland and its duct; *d.l.*, dorsal lamina; *end.*, endostyle; *g.*, gonad; *g.d.*, genital duct; *int.*, intestine; *m.*, muscular mantle; *oes.*, aperture leading from branchial sac into esophagus; *or.s.*, buccal siphon; *ph.*, branchial sac; *st.*, stomach; *t.*, test or cellulose mantle; *tn.*, buccal or coronary tentacles; *ty.*, typhlosole, internal fold of intestinal wall, to increase the digestive surface.

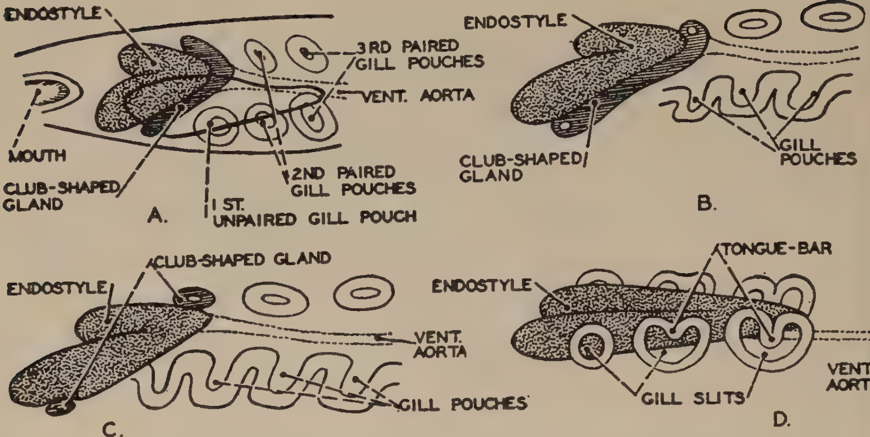

FIGURE 6. Four stages in the development of the endostyle of *Amphioxus*. The evidence indicates that the endostyle is developed from a pair of modified gill pouches. (From NEAL & RAND, *Comparative Anatomy*, Blakiston Co., after VAN WIJHE.)

endostyle (FIGURES 9 and 10) and its transformation into the thyroid gland in the brook lamprey, *Ichthyomyzon*. He recognized six types of cells in the endostyle epithelium, as compared with the five types reported by Marine. Cells of type IV appear to be most active, with type II contributing to the formation of definitive follicles (see FIGURE 11). No comparable anlagen of definitive follicles are present in the endostyle of protochordates.

Beginning, thus, with a diffuse thyroid of few follicles, the gland has become more extensive, retaining its diffuse character in the bony fishes* and becoming encapsulated in the higher forms.

In all forms below the Chordates, direct observation has not disclosed a functional thyroid. However, Murray[63] in 1891 had cured myxedema by the administration of thyroid gland whose active principle was unaffected by drying and heating. It was recalled that Fyfe[64] had long before (1819) discovered the presence of iodine in sponges, and Coindet,[65] a year later, had demonstrated the therapeutic value of iodine in the treatment of goiter and had established it as the active agent in the remedies, such as seaweed and burnt sponge, which had for centuries been found beneficial in the treatment of goiter. In 1848, Vogel[66] showed that sponges contained iodine in organic combination. Further research[67] dis-

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* While this paper was in press, Matthews[204] described a compact encapsulated thyroid gland in 3 genera (*Pseudoscarus guacamaia*, *Sparisoma* sp., and *Scarus* sp.) of Bermuda parrot fish. The thyroid in these fish is large, extending from a point under the base of the tongue to about a cm. anterior to the anterior end of the ventral aorta. The time of development of the capsule and/or other factors responsible for this compact gland remain to be ascertained. Previously, a compact thyroid gland for a teleost, the swordfish, *Xiphias*, had been reported by Addison and Richter.[43] The thyroid of *Xiphias* consists for the most part of 4 masses separated from each other by thin connective tissue septa. In addition, small groups and rows of follicles are scattered in a typical teleostean manner in the loose connective tissue between the chief portion of the gland and the wall of the aorta and the branchial vessel. It would be of interest to learn whether supplementary scattered follicles are present in the parrot fish. Since radioactive iodine was concentrated only by the thyroid gland and by the tissues of the gill region in the parrot fish,[204] it is very likely that thyroid follicles are to be found in this region. The final answer awaits histological study.

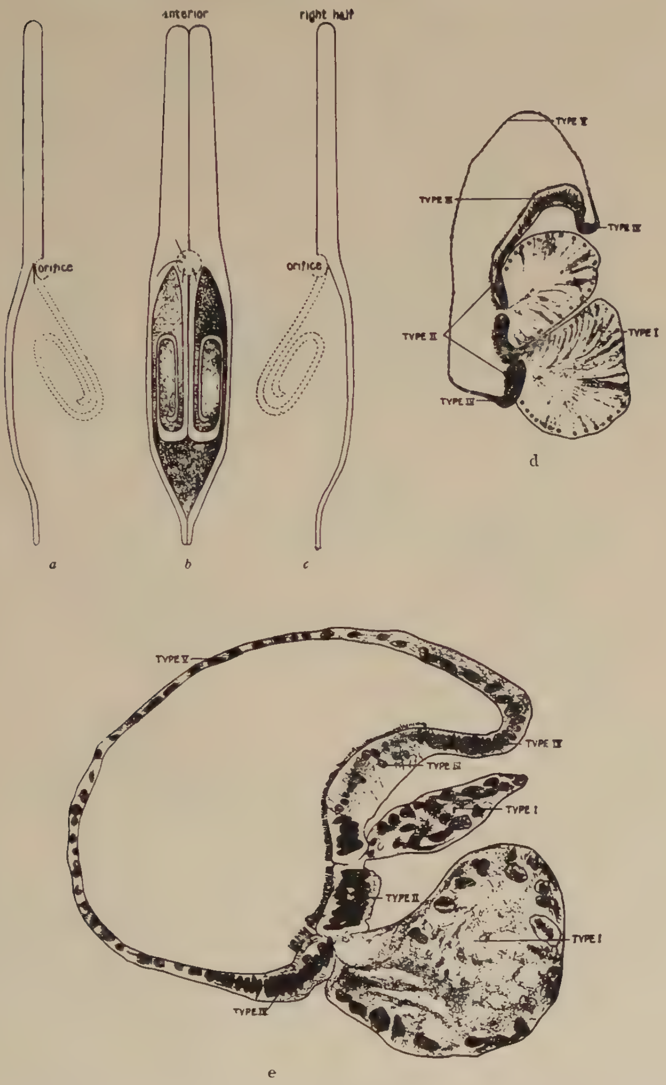

FIGURE 7. Diagrams of the endostyle of *Ammocoetes branchialis* (after MARINE[61]). *a,* Schematic diagram of the left half of the endostyle. The single anterior chamber branches at the anterior edge of the orifice into an inner coiled chamber and an outer coiled chamber. *b,* Composite diagram of right and left halves; the two coiled chambers rotated outward from their normal upright positions. *c,* Schematic diagram of the right half of the endostyle. *d,* Camera lucida outline of a transverse section of the left external chamber posterior to the coil, showing the arrangement and distribution of five types of epithelium in the normal fully developed endostyle. *e,* Camera lucida outline of a transverse section of the left external chamber posterior to the coil, showing the earliest changes in the epithelia at metamorphosis.

closed that tropical and subtropical horny sponges contained as high as 14 per cent iodine in organic combination. At this time, Baumann[68] re-

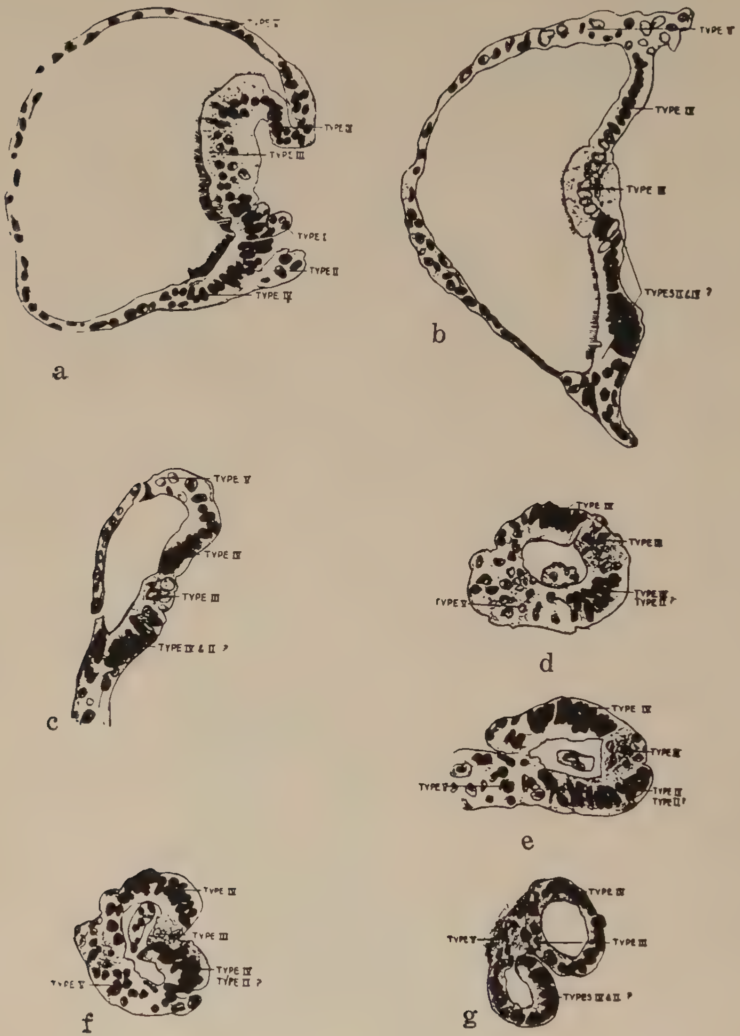

FIGURE 8. Diagrams of the development of thyroid follicles from endostylar cells. (After MARINE.[61]) *a,b,c,* Camera lucida outline of transverse section of left external chamber posterior to the coil showing several stages of metamorphosis. *d,e,* As above, but showing last stages of metamorphosis. *f,* As above, but illustrating the earliest ductless thyroid formation. *g.* As above, but illustrating two distinct follicle formations from single chamber.

ported that he had boiled thyroid glands with 10 per cent sulfuric acid and had separated a physiologically active fraction comprising about 0.2 to 0.5 per cent of the gland substance and containing about 10 per cent iodine. The material was named thyreoiodin and then renamed iodothyrin. Simultaneously, Drechsel[69] isolated an iodine-containing

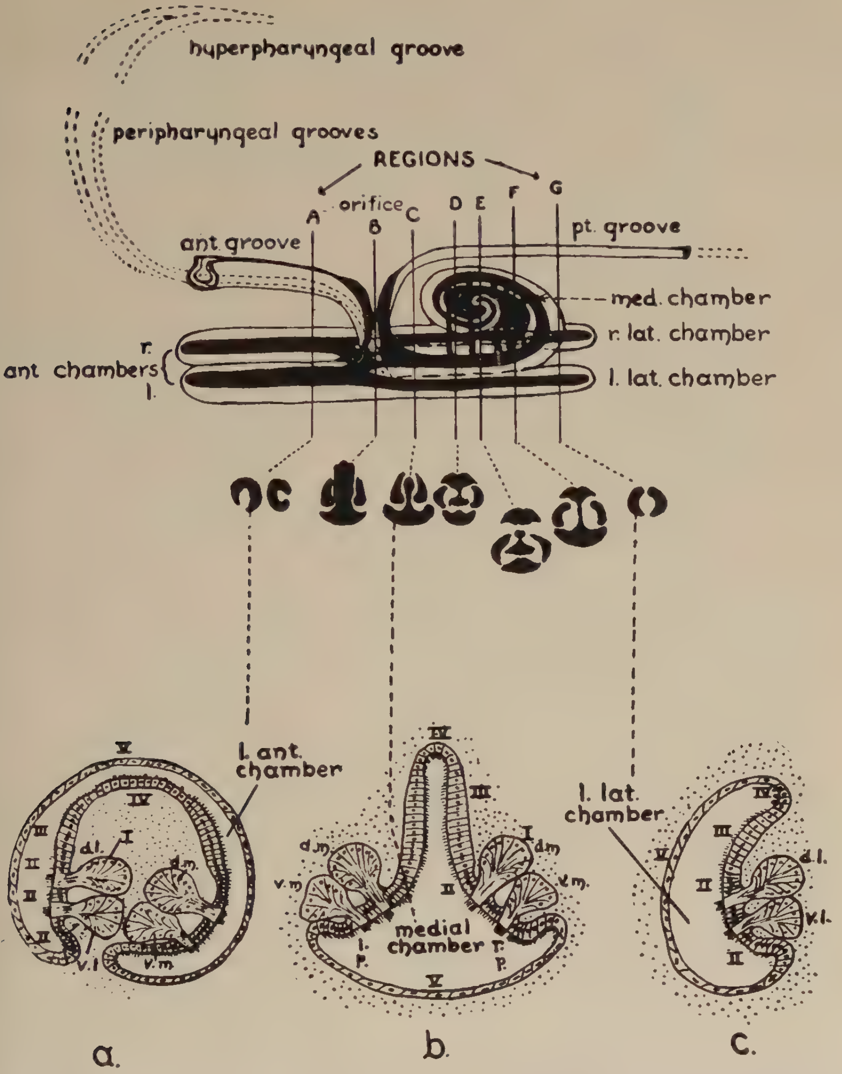

Figure 9. A diagram to show the structural plan of the endostyle. Endostylar chambers are shown in solid black in the upper portion of the figure, and their cross-sectional distribution below the regional lines *A* to *G*. The lower portion of the figure (*a*, *b*, *c*) illustrates the three fundamental types of chambers in relation to the distribution of the types of cells (*I* to *V*). *d.l.*, dorsolateral cylinder; *v.l.*, ventrolateral; *d.m.*, dorsomedial; *v.m.*, ventromedial (bilateral aspects are shown only in *b*); *l.p.*, left pair of cylinders; *r.p.*, right pair. (After Leach.[56])

amino acid—iodogorgonic acid or iodoaminobutyric acid. Following Henze,[70] who had indicated that the substance was not iodoaminobutyric, Wheeler and Jamieson[71] demonstrated that it was 3,5 diiodotyrosine. Similarly, this was also shown to be the iodine complex in the sponge.[72]

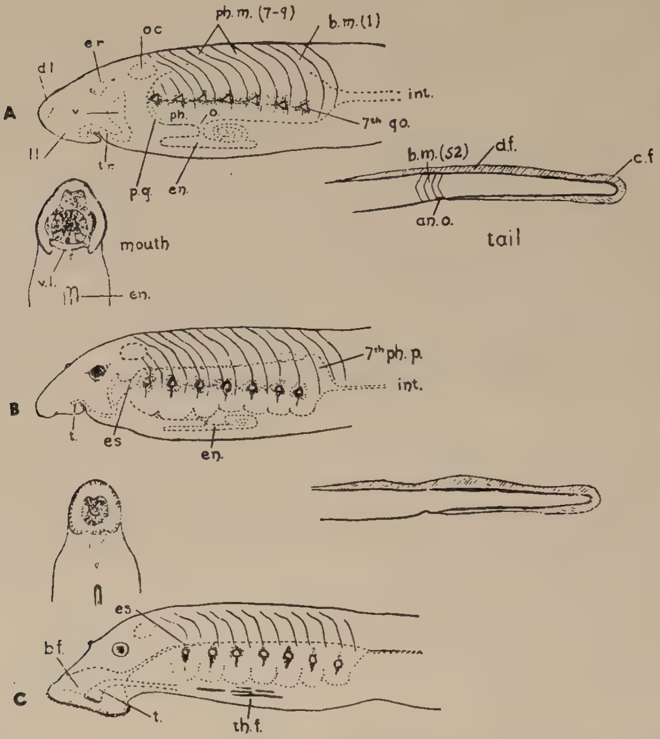

FIGURE 10. The transformation of the brook lamprey, *Ichthyomyzon fossor*. *A*, Morphological features of a fully grown ammocoete. *B*, Changes during early metamorphosis. *C*, Morphological condition of sexually mature male. *an.o.*, anal opening; *b.f.*, buccal funnel; *b.m.*, body myotomes; *c.f.*, caudal fin; *d.f.*, dorsal fin; *d.l.*, dorsal lip; *en.*, *endostyle*; *es.*, esophagus; *e.r.*, eye rudiment; *int.*, intestine; *l.l.*, lateral lip; *o.*, *orifice of endostyle*; *o.c.*, otic capsule; *ph.m.*, pharyngeal myotomes; *p.g.*, *peripharyngeal grooves*; *t.*, tongue; *th.f.*, *thyroid follicles*; *t.r.*, tongue rudiment; *v.l.*, ventral lip; *7th g.o.*, seventh gill opening; *7th ph.p.*, seventh pharyngeal pouch. (After LEACH.[62])

Since that time, iodine analyses[73, 74] have disclosed iodine in all marine animals ranging from traces to as high as 78 gms. per kilogram in the skeleton of the sea fan.

Experimental

In order to ascertain whether animals which normally do not possess a thyroid gland respond to its secretions, it was necessary to resort to experimental techniques. Since the investigations conducted on the invertebrates and protochordates were influenced by those performed upon the Amphibia and mammals, it might be profitable to step out of phylogenetic line and to recall several critical experiments.

Gudernatsch,[75] some 35 years ago, maintained frog tadpoles on a variety of diets and discovered that fresh thyroid glands induced an amazingly accelerated metamorphosis. The hypophysectomy of the frog by

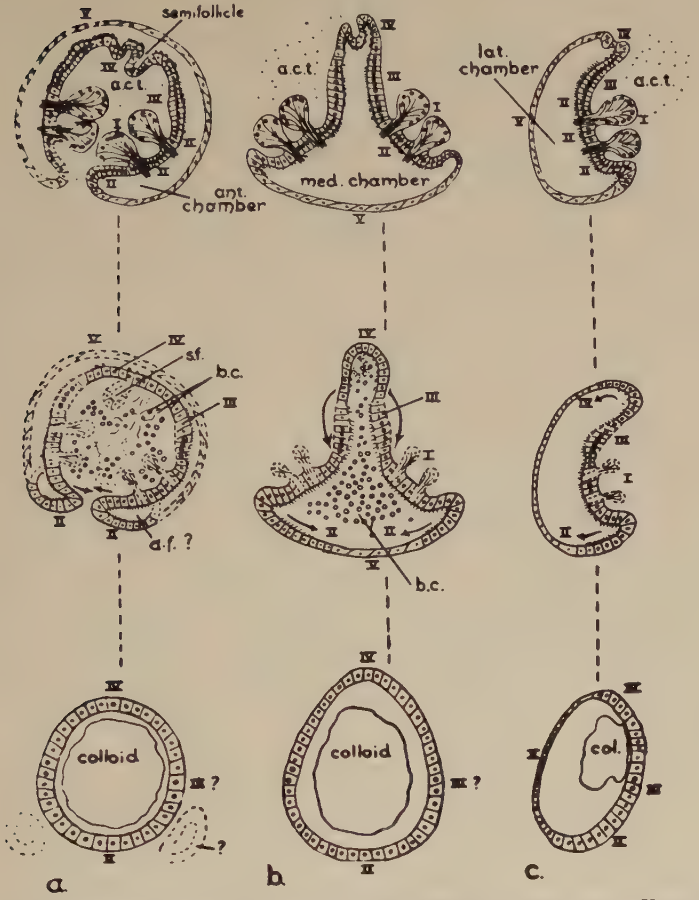

FIGURE 11. The essential features of the development of definitive thyroid follicles. Upper row, diagrams of endostylar chambers of the arrested growth period; middle row, at the end of the histolysis phase of early transformation; lower row, definitive follicles of the sexually ripe adult. *a.c.t.*, areolar connective tissue; *a.f.*, an aberrant type of follicle sometimes seen in early transformation; *b.c.*, blood cells assumed to migrate to the medial follicles; *s.f.*, indicates the fate of the semi-follicles; arrows indicate the realignment of the cell types. (After LEACH.[56])

P. E. Smith[76] and hypophysectomy and thyroidectomy by B. M. Allen[77-79] gave us the now well-known picture of a tadpole which would not go through the various maturation changes leading to the adult frog.

The results of the original feeding experiments of Gudernatsch were so startling that one is apt to forget the picture in the mammal. The failure of the cretin to attain the status of what we regard as the normal adult is evidence in favor of the view that the thyroid plays an important role in the maturation of the mammal. This point Gudernatsch has repeatedly emphasized. With these facts for a background, one may proceed to the experimental phase of the review.

Invertebrates. Examination of the data in TABLE 1 reveals that exposure of Infusoria to thyroxin, thyroid extracts, or thyroid powder yielded controversial results. Cori[83] has suggested that thyroid extract may produce its effect in the invertebrates through some principle other than thyroxin. In his review paper, Schneider[87] concluded that thyroid extract and thyroxin exert an effect on the metabolic processes in the unicellular organism and during the early cleavages of other invertebrates.

TABLE 1

SUMMARY OF EXPERIMENTS OF THYROID ADMINISTRATION TO INFUSORIA

Investigator	*Substance*	*Results*
Nowikoff[80]	Thyroid powder	Accelerated cell division
Shumway[81]	Fresh thyroid powder Iodine, iodothyrin	Accelerated cell division No effect
Buddington & Harvey[82]	Fish, frog, turtle, chick, cat thyroid	Accelerated cell division
Cori[83]	Thyroid extract Thyroxin 1:100,000	Accelerated cell division Questionable
Woodruff[84]	Thyroid powd., thyroxin	No effect
Ball[85]	Thyroid extract	Accelerated cell division
Torrey[86]	Thyroxin 1:100,000–1:1,000,000	Depressed cell division Accelerated catabolism

Thyroid feeding in *Planaria velata* resulted in an apparent speeding-up of metabolic processes and a great reduction in size. This decrease was not due to inanition, since the animals were observed taking the food readily. Moreover, they became smaller than those which were starved.[88] In another species of flatworm, *Planaria maculata*, Goldsmith[89] observed that thyroid feeding did not bring about a reduction in size nor did it alter the rate of head regeneration following a number of successive decapitations. It was, however, observed that the worms on the thyroid diet produced significantly fewer egg capsules than did the animals on other solid diets (TABLE 2).

Little has been done with the annelids. Young polychaete worms were treated with thyroid gland preparations and appeared to show a precocious sexual maturity including the more rapid development of ripened eggs. Growth and regeneration were not affected. Inorganic iodine yielded negative results.[90]

The literature pertaining to the effects of thyroid principle upon insects is replete with contradictions and inconsistencies. The partial summary (TABLE 3) presents the state of our knowledge in this connection. Much of the experimentation is open to criticism in that dosages were not adequately controlled (possible improvement of the ration by the thyroid supplement or possible toxicity of higher concentrations) and the insects were not of known ancestry. In one of the better conceived statistical investigations, Alpatov[102] concluded that thyroid feeding accelerated larval development, with smaller adult *Drosophilae* emerging. In a thorough study of *Tribolium*, Schneider[107] selected optimum dosages of thyroid substance and was able to accelerate metamorphosis of not only the treated larvae but also the untreated larvae of the F_1, F_2, and F_3 descendants.

TABLE 2

NUMBER OF EGG CAPSULES PRODUCED BY *PLANARIA MACULATA* ON INDICATED DIETS

Number of animals	*Food*	*Number of capsules to 4/22**	*Number of capsules to 5/12*
9	Liver gland	68	87
18	Anterior pituitary gland	36	40
17	Anterior pituitary-thyroid gland	55	62
18	Thyroid gland	18	20
4	Liver aqueous extract L	1	1
5	Liver aqueous extract S	0	0
9	Anterior pituitary aqueous extract	0	0
9	Anterior pituitary-thyroid aqueous extract	0	0
9	Thyroid aqueous extract	0	0
9	Starve	0	0

* The animals were decapitated on 4/23. They continued to produce capsules to 5/12. (From GOLDSMITH, E. D. Biol. Bull. 73.)

The work with the crustaceans, molluscs and echinoderms, too, does not enable one to reach a satisfactory conclusion. The bulk of the evidence appears to favor the view that thyroxin does not benefit or accelerate development (TABLE 4).

Ascidians. As was pointed out in the "*Descriptive*" portion of the paper, the ascidian, a protochordate, possesses an endostyle, passes through a larval stage, and undergoes metamorphosis. In the adult, no thyroid gland is recognizable. Here, then, appeared excellent material to test the effects of thyroid substance (TABLE 5). To this end, Weiss,[121] using sea-water solutions of thyroid extracts, hastened the resorption of the tail in *Ciona intestinalis*. That thyroid substance was not specific for this acceleration is to be concluded from subsequent work. 100 per cent metamorphosis was induced in groups of *Ascidia nigra* larvae within 3 hours after hatching by sea-water extracts of pharyngeal, atrial or mantle tissues of adult *A. nigra*, as compared with 30 hours for 100 per cent metamorphosis in untreated controls.[122] The acceleration was even greater in another form, *Polyandrocarpa*. Sea-water extracts of mammalian whole thyroid glands also accelerated metamorphosis, as did cysteine, histidine, leucine, iron and copper salts, and iodine to a lesser degree.[123] Diiodotyrosine, thyroxin, *dl*-phenylalamine, and cysteine yielded negative results. Sea-water extracts from ascidian endostyles acted as very efficient accelerators, but not more so than other ascidian tissues. Brad-

TABLE 3

SUMMARY OF EXPERIMENTS OF THYROID ADMINISTRATION TO INSECTS

Investigator	*Animal*	*Substance*	*Results*
Kunkel[91]	*Lucilia, Calliphora*	Mammalian thyroid excl.	Short. larval & pupation
Romeis[92]	*Calliphora*	Mammalian thyroid excl.	Retardation
Brannon[93]	*Lucilia*	Mammalian thyroid excl.	Short. pupation
Abderhalden[94]	*Deilephila*	Hydrolyzed thyroid	No effect, great variation
Kopeć[95]	*Lymantria, Pieris*	Willow leaves & thyroid	No effect, smaller chrysalids
Magaudda[96]	*Pieris*	Thyroid extracts	Short treatment—acceleration Long treatment—retardation
Romeis[97]	*Papilio*	Inject thyroxin	No effect
Fleischmann[98]	*Lymantria*	Inj. thyrox. 1:10,000	No effect
Fleischmann[98]	*Celerio*	Inj. 0.3 mg. thyroxin	No effect
Kahn[99]	*Tenebrio*	Thyroid substance	No effect
Hahn[100]	*Tenebrio*	1 thyroid: 3 meal	No effect on rate or size
Hahn[100]	*Vanessa*	Nettle leaves & thyroid	No effect on rate or size
Reznitchenko[101]	*Drosophila*	Thyroid 5% ration	No effect
Reznitchenko[101]	*Drosophila*	Thyroid 10% ration	Retardation
Alpatov[102]	*Drosophila*	Thyroid in ration	Short larval, smaller
Koller[103]	*Drosophila*	Thyroxin	No effect
Comas[104]	*Chironomus*	Thyroid extract	Short larval
Zavřel[105]	*Chironomus* late summer	Thyroid extract	No effect on metabolism
Zavřel[105]	*Chironomus* early summer	Thyroid extract	Acceler. growth & metab.
Terao & Wakamori[106]	*Bombyx*	Mulberry leaves & thyr. 1:50-1:400 food	Ate less. Adults smaller. Accel. 2nd generation
Schneider[107]	*Tribolium*	1 thyroid: 5 food	No effect
		1:10–1:100	Accel. to 2nd generation

way[124] obtained no speeding up with crystalline thyroxin in sea-water solutions of 1:10,000, 1:20,000, down to 1:1,280,000. Immersion of larvae in (*1*) isotonic sodium chloride alone at pH 8.2 for one hour followed by normal sea-water, (*2*) sea-water diluted 50% by distilled water for 15 minutes and then transfer to normal sea-water, or (*3*) sea-water containing neutral red 1:50,000 was followed by marked acceleration of metamorphosis. Additional evidence of the non-specificity of the stimulating agent is to be found in the observations of Zinkin[125] in another tunicate, *Botryllus*, in which brilliant cresyl blue, methylene blue, neutral red, strychnine, and potassium bicarbonate in certain concentrations accelerated metamorphosis. These results are summarized in TABLE 5.

Corroborating these findings from another angle, Gorbman[126] has demonstrated that in the ascidians, *Styela* and *Ciona*, as well as in *Amphioxus*

TABLE 4

SUMMARY OF EXPERIMENTS OF THYROID ADMINISTRATION TO *CRUSTACEA*, MOLLUSCS AND ECHINODERMS

Investigator	*Animal*	*Substance*	*Results*
		Crustacea	
Vecchi[108]	*Cyclops*	Dried thyroid	Accelerated development
Banta[109]	*Daphnia*	Dried thyroid	No effect
Romeis[110]	*Astacus*	Thyroid excl. 5 mo.	No effect. Transf.
Ciabetta[111]	*Artemia*	Thyroid extracts	Accel. develop. small
		Mollusca	
Hykès[112]	*Physa*	Thyroxin 1:100,000–1:500,000	Uncertain
Susaeta[113]	*Barnea* eggs	Thyroxin, low & high	Retard. development
Susaeta[113]	*Barnea* eggs	Thyroid 5 cc/liter	Slight acceleration
Susaeta[113]	*Barnea* eggs	Thyroid 5 cc+/liter	Retard. 2 & 4 cell suscep.
Chatzillo[114]	*Limnaea*	Thyroid	Accel. develop. in 60%
		Echinoderm	
Butler[115]	*Arbacia*	Thyroxin 1:25,000	Retard. 1st cleav. 10 min.
		Thyroxin 1:50,000	Retard. 1st cleav. 5 min.
		Thyroxin 1:100,000	Retard. 1st cleav. 5^- min.
Torrey[116]	*Echinometra*	Thyroxin 1:50,000–1:1,000,000	Slight to comp. inhibition
Runnström[117]	*Antedon*	Thyroxin	Retardation of cleavage
Zavřel[118]	*Paracentrotus*	Thyroxin	Retardation of cleavage
Ungar[119]	*Psammechinus*	Thyroxin	No effect 1st cleav. stages
Ungar[119]	*Psammechinus morula*	Thyroxin 1:50,000–1:800,000	Retarded development
Ungar[119]	*Psammechinus morula*	Thyroxin 1:850,000–1:2,000,000	Accel. development

TABLE 5

SUMMARY OF EXPERIMENTATION ATTEMPTING TO ACCELERATE METAMORPHOSIS IN ASCIDIANS

Investigator	*Recipient*	*Treatment*	*Result*
Spaul[120]	Frog	Ascidian endostyle, pharynx, mantle	—
	Frog	Dogfish thyroid	+
Weiss[121]	Ascidia	Immersion & feeding thyroid	+
Grave[122]	Ascidia	Sea-water extract pharyngeal, atrial, mantle tissue of adult Ascidia	+
	Ascidia	Sea-water extract mammalian thyroid	+
Grave & Nicoll[123]	Ascidia	Cysteine, leucine, histidine	+
		Cysteine, tyrosine, diiodotyrosine, *dl*-phenylalanine	—
		Fe, Al, Cu, I	+
		Sea-water extract ascidian endostyle, other tissues	+
Bradway[124]	Ascidia	Thyroxin 1:10,000, 1:20,000 to 1:1,280,000	—
		I 1:80,000,000	+
		Immersion 15 min. sea-water dil. 50% distilled	+
		Neutral red in sea-water 1:50,000	+
Zinkin[125]	Ascidia	Brilliant cresyl blue, methylene blue, neutral red, strychnine, potassium bicarbonate	+

the endostyle did not accumulate radioactive iodine. Iodine was picked up and stored in the stolonic septum (FIGURE 12) to a degree fully as great as the vertebrate thyroid.[127] Previously, Marine[60] had found 3.63 mg. iodine per kilogram in the tunic, with 0.03 mg. per kilogram in the rest of the body, but none in the ascidian endostyle. The significance of the high concentration of iodine in the stolonic septum and tunic escapes us at this time.

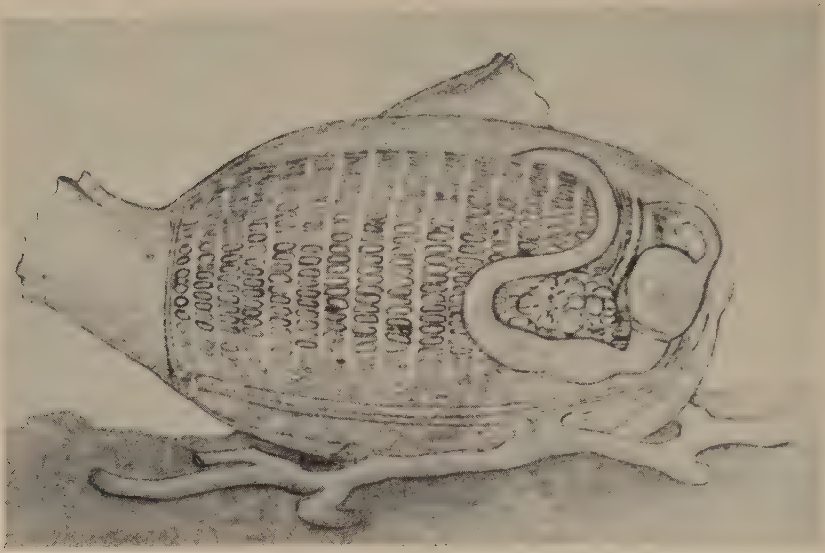

FIGURE 12. Ascidian, *Ecteinascidia*, from left side. The growing stolon is shown at base.

Cyclostomes. Considerable evidence of a morphological nature has been presented in support of the view (see "*Descriptive*") that the lamprey thyroid arises from the larval hypobranchial groove. Additional evidence is to be found in the radioactive iodine work of Gorbman and Creaser.[128] The lamprey, *Entosphenus*, localized radioactive iodine in the endostyle and kept it there for one to five years, *i.e.*, for the duration of the larval period. It is of interest to note that the type III cells were found to be the principal accumulators of iodine, with smaller amounts demonstrable in type V, and negligible quantities or none at all in types I, II, and IV (FIGURE 13). The reason for the discrepancy between these observations and those of Marine and Leach is not at all clear. In FIGURE 14, one may see the uptake of radioactive iodine by the developing thyroid gland of the frog.[129]

Spurred on by findings in the Amphibia and developments in the treatment of myxedema, attempts were made to alter the rate of metamorphosis of the lamprey by exposure to substances of known potency. A number of *Ammocoetes* had been immersed in sea-water containing

Lugol's solution, but no modification of the endostyle could be recognized.[60] Analyses of ammocoete endostyles by Kendall's method,[130] accurate to 0.005 mg., revealed no iodine.[131] In TABLE 6, additional data are presented. It would appear that the lamprey thyroid contains the active thyroid principle which can be detected by the Gudernatsch tadpole test, but the ammocoete transformation is not affected by those substances known to be potent accelerators in the amphibian.[132–135] It is true that the thyrotrophic factor of the pituitary appeared to bring changes in the endostyle cells,[126, 136] but hypophysectomy was not followed by any detectable change in the endostyle.[136] Iodine and thyroid had no effect upon ammocoete respiration.

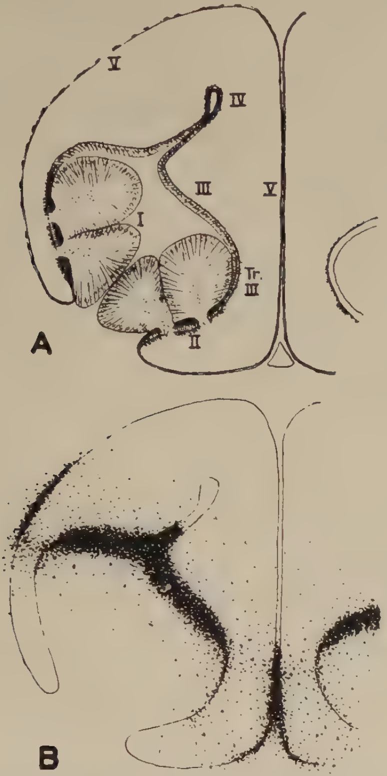

FIGURE 13. Determination of distribution of radioactive iodine within endostyle of lamprey. *A* is a diagram of cell types in a section of an anterior chamber of the endostyle. *B* is the radio autograph made by this section projected on a tracing of the same section. In this way the cell types in which iodine has been accumulated are identified. (From GORBMAN & CREASER.[128])

In view of these observations, the conclusion of Horton that the thyroid gland has no significance in the metamorphosis of the lamprey still appears to be valid.

Fish. The experimental data dealing with the role played by the thy-

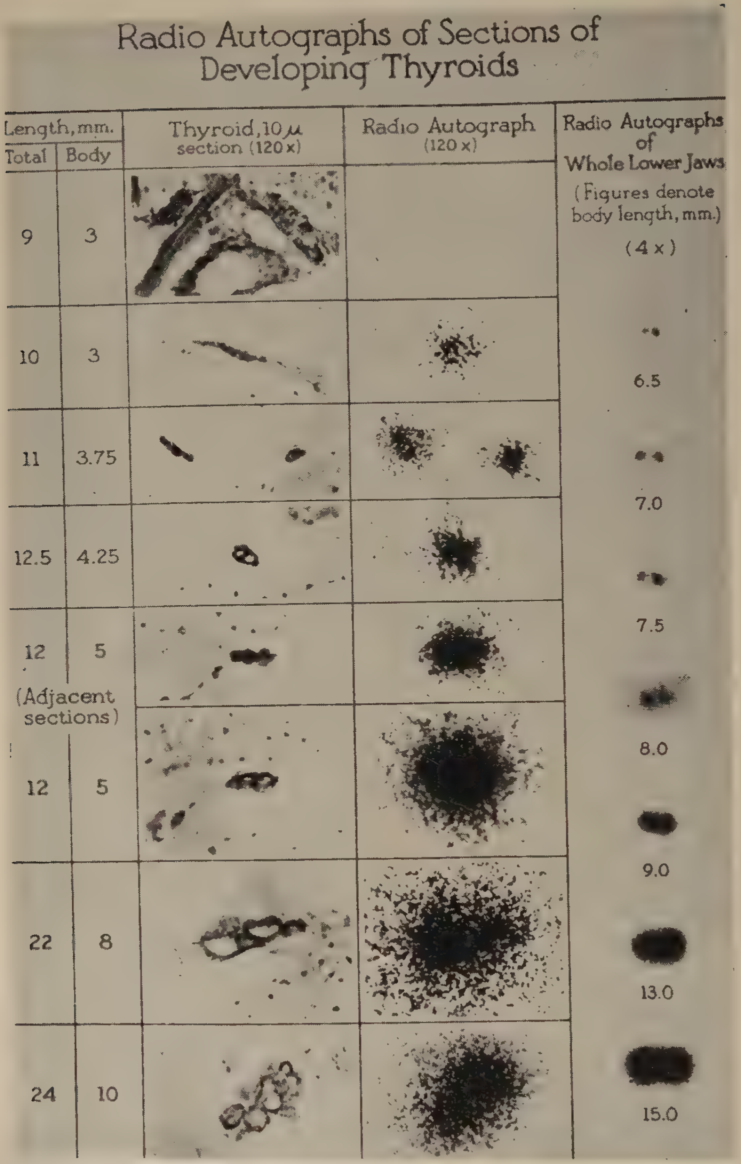
Radio Autographs of Sections of Developing Thyroids
Length, mm.
Total
Body
Thyroid, 10 μ section (120 x)
Radio Autograph (120 x)
Radio Autographs of Whole Lower Jaws
(Figures denote body length, mm.)
(4 x)
9
3
10
3
11
3.75
12.5
4.25
12
5
(Adjacent sections)
12
5
22
8
24
10
6.5
7.0
7.5
8.0
9.0
13.0
15.0

FIGURE 14. First faint traces of storage of radioactive iodine in thyroid of 10-mm. (total length) tadpole. At this stage each of the two thyroid lobes consisted of 3 to 6 follicles. (From GORBMAN & EVANS.[129])

roid gland in the physiology of the fish have been negative or contradictory. No one, to my knowledge, has been able to alter the oxygen consumption by the administration of the thyroid substance or thyroxin.[137–]

TABLE 6

SUMMARY OF EXPERIMENTATION ATTEMPTING TO ACCELERATE METAMORPHOSIS IN LAMPREYS

Investigator	*Recipient*	*Treatment*	*Result*
Jensen[132]	Ammocoetes	Inject iodothyrin	—
Rémy[133]	Ammocoetes	Inj. & immersion (26 d.) iodothyrin	—
Horton[131]	Ammocoetes	Iodoform, I, KI, sheep thyroid extract	—
	Frog	Ammocoetes endostyle, frog muscle	—
	Frog	Lamprey, ox thyroid	+
Young & Bellerby[134]	Ammocoetes	Inject anterior pituitary	—
Stokes[135]	Ammocoetes	Thyroxin 1:500,000	—
		Thyroid 1:120,000–1:500,000	—
Knowles[136]	Ammocoetes	Inject thyrotrophic factor	Accel. changes endostyle cells
	Ammocoetes	Hypophysectomy	No change endostyle
Gorbman[136] (unpub.)	Ammocoetes	Inject thyrotrophic factor	Hyperplasia endostyle cells (III)

[139] That the oxygen consumption can be raised was demonstrated by Hasler and Meyer, who caused an increase by the injection of 2–4 dinitrophenol.

Histological studies have revealed a seasonable change in the structure of the thyroid glands of several teleosts.[45, 140, 141] In the eel,[44, 142] the thyroid appears to be involved in the metamorphosis. The fish, *Periophthalmus*, metamorphoses into a partly terrestrial animal, which transformation, in the opinion of Harms,[143] is under the influence of the thyroid. Hoar, in his analysis of the changes in the thyroid gland of the salmon, described a definite positive correlation between periods of intense growth and tissue changes. Young salmon undergo marked changes before entering the sea, and this transformation has been compared with that of the metamorphosis of the eel and amphibian. The early observations of Gudernatsch,[40] that the epithelium of the thyroid decreases in height and the colloid becomes acidophilic with age, have been confirmed in the salmon and interpreted[45] as evidence that the gland is more active in the young fish. Ferguson's[144] observation that the size of the follicle appeared to be proportional to the size of the fish has been corroborated in the salmon and interpreted as an indication that the gland had been active at some previous time.[45]

The administration of mammalian thyroid substance to several species of fish has produced alterations in growth and maturation.[140, 143, 145, 146] These effects have been in the nature of a decreased growth rate with altered body proportions, exophthalmos, precocious and atypical growth of the anal fin. Another group of investigators[139] was unable to produce any change in one of the species, *Lebistes*, by the use of thyroid powder mixed with the food in a 1:1 ratio or by the addition of thyroxin to the water. In the light of Gudernatsch's[147] observations following thyroid feeding to the rat, the variability of the above results may be due to the differences in dosage.

The goldfish thyroid was found to respond readily to pituitary extracts from frog, chicken and sheep,[148, 149] and that of *Fundulus* was activated by sheep pituitary extract.[150] Pituitary extract from the sole affected the goldfish thyroid very slightly, but this was found to be due to a low titer of thyrotrophic factor and not an inability of the target organ to respond.[149] Proof for the presence of an active principle in the teleost and elasmobranch thyroid glands is provided by the acceleration of amphibian metamorphosis following transplants[120, 151, 152] and feeding[205] of these glands. A condition simulating Graves' disease consisting of exophthalmos, muscular weakness, thyroid hyperplasia, and emaciation occurring in trout was overcome by the addition of iodine to the water.[153]

To the best of my knowledge, the critical experiment involving thyroidectomy had not been performed. The isolated follicles and their diffuse distribution make a successful operation extremely difficult. Following the successful use of thiourea and its derivatives as antithyroid agents,[154–166] it occurred to us[167] that chemical thyroidectomy was the answer to a problem of long standing.

In individuals of a *Platypoecilus* × *Xiphophorus* strain immersed in 0.03% solution of thiourea in conditioned water for periods up to 70 days, a retardation of growth, failure of development of secondary sex characters (FIGURES 15 and 16), and a marked hyperplasia and hypertrophy of the thyroid were observed by Goldsmith, Nigrelli, *et al.*[167] The growth retardation was not attributed to inanition, since the treated animals accepted food as readily as did the controls. In another series, of *Lebistes*, Nigrelli, Goldsmith, and Charipper[168] found that small amounts of mammalian thyroid powder added to the diet of the thiourea-treated animals resulted in fish which could not be distinguished from the controls. The inactive thyroids of these animals resembled those of untreated animals, as contrasted with the hyperplastic ones of the fish exposed to the goitrogen. Representative sections of these glands are to be found in FIGURES 17 and 18.

From the combined evidence presented here, it would appear reasonably clear that the thyroid gland functions in the maturation of the fish although it may have no calorigenic function.*

From this point upward, the story is straightforward. The reviews by Allen,[79] Gudernatsch,[169] Schneider,[87] Gorbman,[170] and Adam[171] make an extensive survey unnecessary. The more recent experimentation utilizing the goitrogens, thiourea, and thiouracil provides additional evidence as to the phylogenetic relationship of the vertebrate thyroid.

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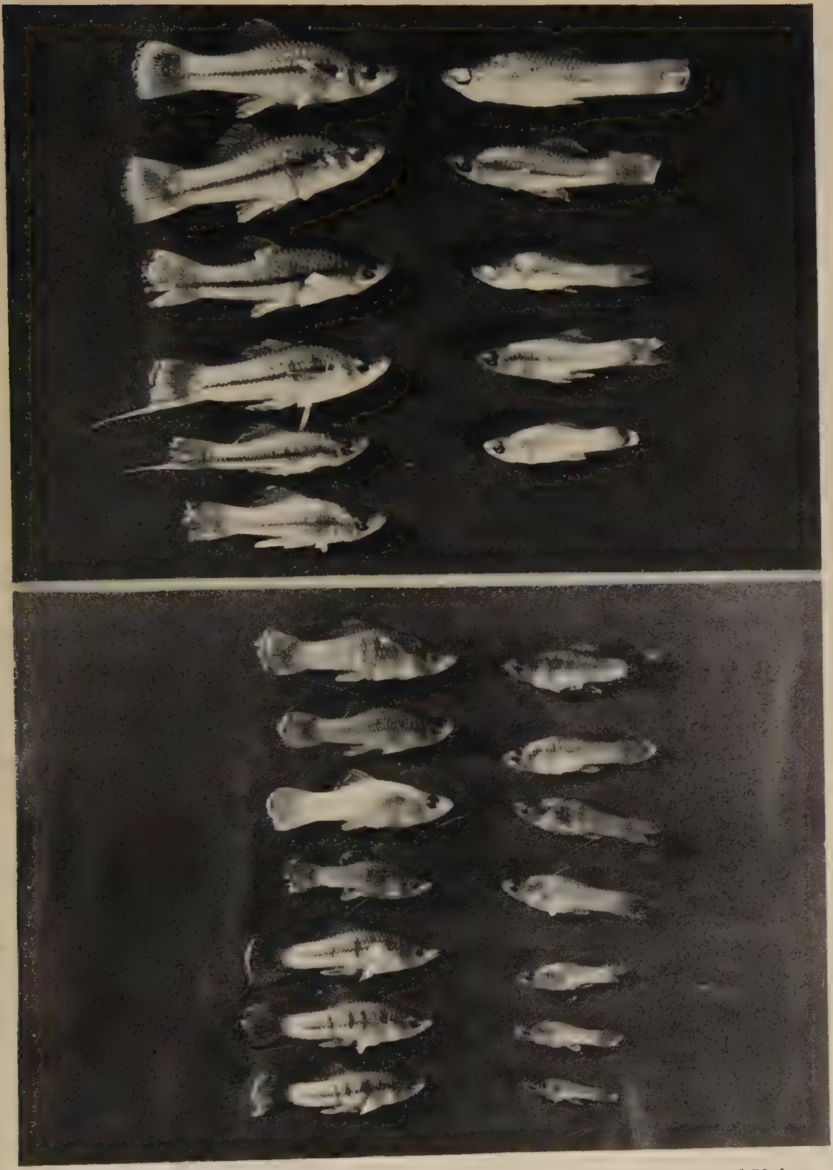
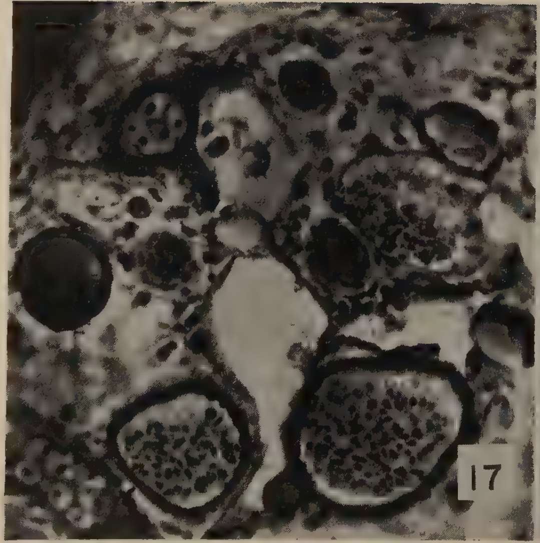
* While this paper was in galley, Smith and Matthews[206] reported that injection of extracts made from the thyroids of Bermuda parrot fish, *Sparisoma* sp., into white grunts, *Bathystoma* sp., resulted in an increase of oxygen consumption in those fish weighing more than 15 grams. Several of the grunts failed to respond and in the light of the weight of negative evidence it would be profitable to repeat the work using greater numbers of fish.



FIGURES 15 and 16. *Platypoecilus* x *Xiphophorus* hybrids immersed in thiourea for 79 and 73 days, respectively. Note the size and differentiation differences between the untreated controls on the left and the thiourea-treated on the right. (After unpublished figure of GOLDSMITH, NIGRELLI, GORDON, & CHARIPPER.)

Amphibia, Birds, and Mammals. Goldsmith and his collaborators[157, 164] inhibited metamorphosis of the frog tadpole by immersion in or in-



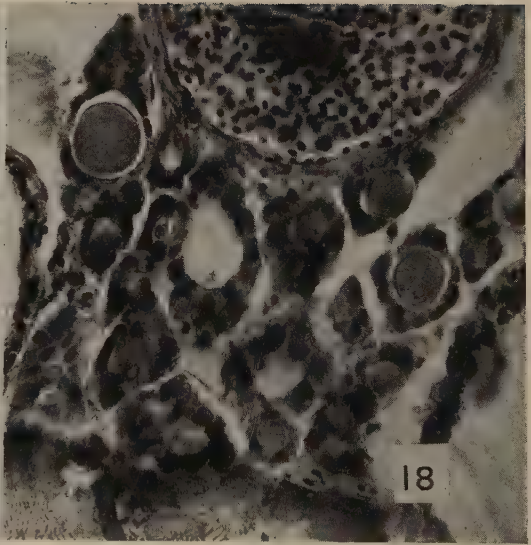
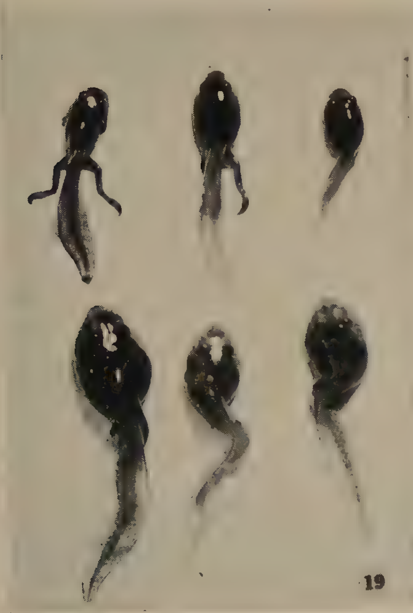


FIGURE 17. Representative section of thyroid gland of untreated *Lebistes reticulatus*. The number of follicles is few and the epithelium is flat. The picture in the fish receiving thiourea-thyroid powder supplement is similar.

FIGURE 18. *Lebistes reticulatus* immersed in thiourea for 4 months. Thyroid gland is hyperplastic, showing abundance of follicles in single field. Widespread proliferation with invasion into branchial region (not shown) is common. Epithelium is much higher than that of control. (Both figures after NIGRELLI, GOLDSMITH, & CHARIPPER, unpublished.)

jection of thiourea. Growth, however, continued with the development of giant tadpoles (FIGURES 19 and 20), reminiscent of those obtained by Allen[78] and Hoskins and Hoskins[172] following complete thyroidectomy. Ossification processes were significantly retarded, resembling the effects on the skeleton reported in the thyroidless tadpole.[173] The thyroid picture was one of extreme activation—marked hyperemia, columnar epithelium, scanty colloid. The thiourea effect could be negated by exposure to thyroxin but not by the administration of thyrotrophin. Return of short- and long-term thiourea-treated animals to water was followed by a resumption of metamorphosis and a return to the normal histological pattern in the thyroid.[164] Thiouracil has also counteracted the action of the thyrotrophic factor when administered to tadpoles.[161]



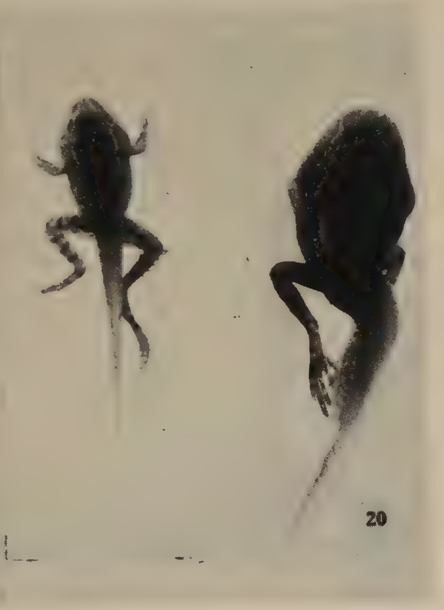


FIGURE 19. Note the larger size, arrested development, and crooked tail base condition in the tadpoles immersed in 0.03 per cent thiourea for 58 days since the 20–30 mm. total length stage (lower animals) as compared with normal *Rana pipiens* larvae in several stages of development (top animals).

FIGURE 20. Recently metamorphosed *Rana pipiens* larvae. Animal on the left is a normal frog 104 days old. Animal on the right had been in 0.03 per cent thiourea for 112 days since the 25 mm. total length stage and then returned to water for 102 additional days (chronological age, 232 days). Note the marked difference in size. (After GORDON, GOLDSMITH, & CHARIPPER.[164])

Dwarfism in chickens analogous to human myxedema infantilis has been reported by Landauer.[174] These observations, those by Schwarz[175] and many others (see bibliographies[87, 175]) have firmly established the importance of normal function of the thyroid for the normal development of the skeleton and plumage of the fowl. Here, too, investigators seized upon the goitrogens, thiourea and thiouracil, and it soon became apparent that the chick thyroid was responsive to these chemicals[177, 178, 179] when given in the proper dosage (0.1%) for suitable periods of time (about 14 days).

A wealth of evidence has been accumulated demonstrating that syndromes comparable to myxedema could be produced in many mammals by thyroidectomy. The pertinent facts in the literature may be found summarized in the reviews by Hammett[180] and Schneider.[87] Suffice it to say that thyroidectomy in the immature mammal has long been known to be followed by a retardation of growth—sheep and goats,[181, 182] rabbits,[183] rats.[184–186] More recently, it has been reported[187–189] that surgical removal of the thyroid one to three days after birth results in a marked retardation of growth and maturation in the rat.

By incorporating thiourea in the diet of the pregnant rat, Goldsmith, Gordon, and Charipper[158, 163] thyroidectomized rats prior to birth. Following parturition, the rats received the goitrogen from their mother's milk and, after weaning, directly from the thiourea diet. In a case where the female had been treated throughout the entire gestation period, two scrawny female young were born. Treatment was continued and they developed at a greatly retarded rate, exhibiting definite cretin characteristics. At the age of 84 days, one weighed 50 gm. and the other 78 gm. as compared with 160 gm. for the control females (FIGURE 21).

The thyroid glands of all treated animals appeared enlarged and hy-

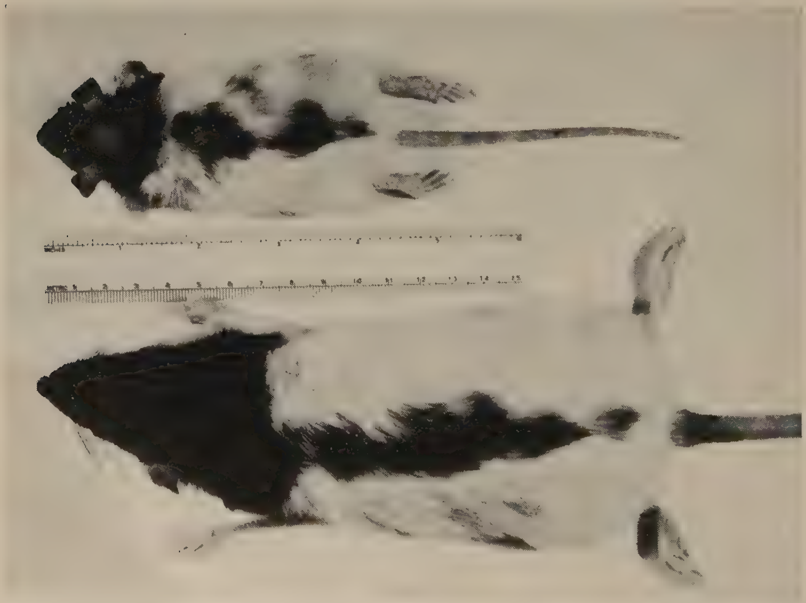


FIGURE 21. Thiourea and growth. 84-day-old thiourea-treated animal (50 gm.) as compared with an untreated (160 gm.) of the same age. The cretin was obtained from a mother treated with thiourea during the entire gestation period. (After GOLDSMITH, GORDON, & CHARIPPER.[163])

peremic, macroscopically; microscopically, irregularity of the follicles, high columnar epithelium, and limited quantities of lightly staining colloid were to be seen. The thyroid of the 50-gm. cretin weighed 50 mg. as compared with a 12 to 15 mg. gland, which is typical for our untreated rats. Somewhat similar results have followed injection of thiouracil in one-day-old rats.[190] Several recent papers pertaining to the human[191–193] have confirmed the above findings.

The similarity in results obtained in all classes of vertebrates following the administration of thiourea and thiouracil lead one to adduce that the synthesis of thyroid hormone is similarly inhibited in all of them, as was first demonstrated in the mammal by Keston, Goldsmith, *et al.*[166] and by Chaikoff and his collaborators.[194]

Reptiles. Comparatively little has been done with the reptile. What facts there are, however, are of a positive and uniform nature and in agreement with the substantial data which have been amassed for the amphibian and mammal.

The lizard[195] and snake[196] pituitaries have been altered following thyroidectomy, with an increase in the size and number of vacuolated basophiles in the latter. Hypophysectomy in the snake[197, 198] is followed by a change from the cuboidal to the squamous type of epithelium in the thyroid. Administration of anterior pituitary activated both normal (resting)[199] and hypophysectomized snakes.[198] The fence lizard thyroid[149] responded to injections of frog, chicken, and sheep pituitaries. Here, too, the sole pituitary was ineffective. Thyroids of *Anolis* treated with estrogens were found to be more active histologically than those of untreated controls. This was correlated with an accumulation of a colloid-like (gonadotrophic or thyrotrophic hormone) material in the pituitary.[200]

Thyroid and Tissues in General. A mass of data has been accumulated[87] in support of the view that thyroid extract or thyroxin applied to isolated vertebrate, invertebrate, and plant tissues accelerated either anabolism, catabolism, or both. That mammals may produce extrathyroidal hormone may be adduced from the observations of a number of investigators.[201–203] It is true, further, that a number of the invertebrates do produce diiodotyrosine, but to the best of our knowledge synthesis of the thyroid hormone takes place for the first time in the adult lamprey gland, and it is in the lamprey that we are able to trace the development of the definitive follicles from endostyle cells.

Recapitulation

Classical developmental studies and experimentation utilizing thyroid and anterior-pituitary preparations, antithyroid agents, and radioactive tracers have resulted in the accumulation of considerable evidence strongly indicating a phylogenetic relationship of the thyroid gland from the lamprey to man. Further, they support the view that there is a common factor underlying thyroid function in the vertebrates.

Thyroid gland substance, when used in suitable concentrations, appears to influence metabolism in isolated tissues of the vertebrates and in the invertebrate animal.

Below the cyclostomes, where no recognizable thyroid tissue has been described, the possibility of thyroid hormone synthesis remains unanswered.

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Discussion of the Paper

DR. FREDRICK GUDERNATSCH (*New York, N. Y.*):

I should like to comment and elaborate somewhat on some of the interesting points brought out in Dr. Goldsmith's presentation.

(*1*) In reference to the primary connection of the developing thyroid with the pharynx, as still seen in even the highest vertebrates, including man, there exists a considerable amount of anatomical and embryological data indicating that all glands now of internal secretion have their phylogenetic ancestry in former glands of external secretion, *i.e.*, all glands now "ductless" once had an open secretory duct. The term "thyroglossal duct" is an appropriate anatomical term. Its occasional developmental anomalies (*e.g.*, the persistence of its lumen) as well as certain pathological structures arising from it (*e.g.*, cysts, neoplasms) are well known.

Thus, the thyroid, too, at one stage of vertebrate ancestry existed as a "duct-gland," most likely as a gland auxiliary to the metabolic functions of the alimentary canal, since it poured its secretion into the lumen of the latter. It has retained this metabolic function even after having lost its direct connection with the digestive tract.

An analogous situation existed at one time in reference to other endocrine structures which now are "ductless glands."

(*2*) All endocrine glands consist of numbers of functional units (isolated cells, clusters of cells, islands, follicles) which have no other connection with each other except by means of the interstitial connective tissue stroma, usually surrounded by a connective tissue capsule. The units are independent of each other functionally, in some glands also anatomically (for instance, chromaffin nodules, pancreatic islands, parathyroid nodules, etc.). Dispersion is a primary characteristic feature of all endocrine structures.

Dr. Goldsmith showed some pictures of the wide dispersion of thyroid follicles in teleost fishes. In some species of the latter, the entire gill region ventrally and laterally to the pharynx is dotted with follicles entirely separate from each other. As he pointed out, there is, in these forms, no connective tissue capsule by which one could anatomically define the "thyroid gland." Structurally speaking, there is no "thyroid gland" in these lower vertebrates, but there are many thyroid follicles.

This is the primary phylogenetic condition, a semblance of which is noticeable during the early embryonic period of all, even the highest vertebrates. The capsule is a later acquisition. Its late formation explains why accessory thyroid nodules are nothing abnormal and why one often finds "foreign tissues," such as parathyroids, muscle fibers, ganglia, etc., within the confines of the human thyroid.

Dr. Goldsmith also referred to the fundamental studies by Marine on thyroid overgrowth in some teleost fishes (trout) and the influence of iodine in checking and reducing such overgrowth. In view of the dispersive state of the thyroid in these forms, one has difficulty in deciding by what degree of dispersion the normal state changes to the pathological. Unless there is extensive formation and spreading of new follicles (which Marine found to a most impressive degree), one is uncertain when to name the spreading a hyperplasia of the teleost thyroid. Still more hazardous would be the designation "neoplastic" thyroid, unless one finds pathological criteria other than mere increase in number and spreading of follicles.

As shown by Marine, the fish thyroid becomes more dispersed (hyperplastic) under the lack of iodine (combined with some other accidental factors). The reverse, involution, takes place under improved conditions. In the encapsulated thyroid of the human, dispersion of follicles is impossible, but hyperplasia occurs just the same. With the formation of new follicles and the general enlargement of all, this hypertrophy often continues to a very impressive degree (formation of a goiter).

(*3*) Dr. Goldsmith has given a very logical picture of the endostyle found in the lower vertebrates as being the early phylogenetic structure from which later the thyroid gland may have developed. In general, anatomists and embryologists are agreed on this, though there is a difference of opinion as to whether in all species which possess them the structures called "endostyles" are homologous among themselves and, in turn, comparable to a primary ancestral anlage of the thyroid.

Why does the anatomist make so much of this endostyle-thyroid relationship and why does he search, in general, for the ancestry of endocrine structures further and further down in the phylogenetic scale, now even in the invertebrates? He is looking for the continuity of these organs throughout the entire phylogenetic tree. In most instances, this anatomical continuity is well established, especially for the thyroid. But even if it were not, even if our present interpretation of the endostyle were incorrect, we have a well-established chemical (which means physiological) endocrine continuity throughout the vertebrates—as regards "thyroid chemistry" even throughout the invertebrates. (Sugar-reducing substances, estrogen-like and other compounds resembling the hormones of higher forms, have also been found in invertebrates. Their specific function there, if any, is not known.)

Iodine is present in all invertebrates examined. Naturally, it would be present in all marine forms. Specific iodine-amino acid combinations, likewise, are present in invertebrates. One of the essential constituents of the hormonal complex of the thyroid, 3,5-diiodotyrosine, was first isolated from one of the lowest invertebrate forms, *viz.*, some species of the Gorgoniidae (corals such as those used for jewelry). It was then named iodogorgonic acid (Drechsel). Around 1905, Lafayette B. Mendel isolated 3,5-diiodotyrosine from Florida sponges, the lowest forms of invertebrates just above the Protozoa. Thus, the point emphasized by Dr. Goldsmith that there is phylogenetic continuity as regards the "thyroid complex" from the lower forms up is well taken. Even though we may not be able to uncover morphological evidences of such continuity, we find chemical continuity throughout, some iodine always being attached to some protein constituents. How far down in the phylogenetic scale such forerunners of thyroid hormonal compounds exert a physiological action resembling that of the true hormone in the higher forms, is unknown. Dr. Goldsmith emphasized correctly the "physiological interchangeability" of hormones across the species borders, meaning that certain hormones obtained from one species, high or low, will produce their principal effects in another species, no matter how high or low (endocrine therapy in man very often makes use of hormonal substances obtained from other forms).

(*4*) Dr. Goldsmith discussed the influence of thyroid material (mammalian material effective in amphibians) in the accelerated metamorphosis of tadpoles and stated that this phenomenon demonstrates the specific influence of the thyroid on the maturation process of the juvenile organism. During metamorphosis, the immature tadpole (the amphibian "paedoform") progresses toward the mature form, the permanent adult frog. The concomitant anatomical changes are very drastic in these lower animals, especially when hastened by the administration of thyroid material. At the same time, pronounced changes in the physiology of these forms take place.

In the human, too, we observe a series of such anatomical and functional changes when the organism progresses from the paedo- to the mature form. These changes, occurring in man during the period of adolescence, are more gradual than those in lower forms where the body changes over completely from one anatomical type to another; yet, they are "metamorphic" changes just the same (body configuration, skeletal changes, secondary sex characters, etc.). As in the advance from tadpole to frog, in the human, too, these changes are brought on, and in retarded cases accelerated, by hormonal factors, a properly functioning thyroid being their prerequisite. That this is the case may be judged from the study of "subnormal" adolescent individuals. If unaided, an athyroid individual will remain permanently infantile (in fact, even below the normal paedoform stage), just as an athyroid tadpole will never change into a frog. Hypothyroid individuals will develop to a state of "maturity" commensurate with the degree of their hypothyroidism. By thyroid medication (usually coupled with pituitary and gonadal hormones), the physician succeeds in bringing such individuals forward to a more advanced stage of maturity, even though they may not be able to reach the functional adult stage. The earlier in life such treatment is initiated, the better the chances for advancement, showing clearly that the thyroid exerts its *maturing* influence during the *entire* period of development, and that it is capable of accelerating the rate of development very early. A tadpole can be changed by thyroid application into a frog at the age of 15 days, while normally it would metamorphose at 75 to 80 days.

In adolescent humans who fail to go through the final steps to full maturity because of lack of thyroid drive, maturation can easily be accelerated and completed by thyroid medication. Closure of various epiphyses, growth of facial and pubic hair, development of breasts, onset of menstruation, descent of testicles in cryptorchids when there is no anatomical impediment in the inguinal canal, are signs of this transformation and maturation.

It is appropriate, therefore, as Dr. Goldsmith has emphasized, to consider the thyroid hormone as a *maturation hormone*, at least in the particular phase of thyroid function here discussed.

CYTOLOGICAL AND CYTOCHEMICAL BASES OF THYROID FUNCTION*

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THE thyroid gland is the only one among the endocrine system which, at the same time that it synthesizes its secretion, stores it in great quantities outside the cells in follicular cavities. This double function of synthesis and storage determines the architecture of the organ, whose anatomical unity is the thyroid follicle.

For these reasons, any study of the cytological and cytochemical foundations on which thyroid function might be based is more complex than in other endocrine glands. Here, one has to consider not only the processes by which the hormone is synthesized, secreted, and stored, but also the mechanisms by which this hormone is reabsorbed and enters into the circulation to be utilized by the organism. As the present Conference demonstrates, this study has progressed a great deal in the last few years, due to the introduction of many new methods of research belonging to the various fields of anatomy, physiology, biochemistry, and biophysics. In this paper, I shall refer particularly to results of the use of cytological and histochemical methods, and especially to the work carried on by us since 1941 at the University of Buenos Aires.

The Intracellular Colloid. Cytological methods have contributed a great deal to the knowledge of the function of endocrine organs but in many cases have failed to show the intracellular secretory processes. Sometimes this is due to the lack of appropriate or specific methods for detecting the products of secretion. In other cases, processes may occur exclusively in the submicroscopic level and the biochemical phenomena are not revealed by visible changes under the microscope.

In the case of the thyroid gland, the literature from 1888 until 1939 reveals great confusion, not only concerning the elements which compose the intracellular colloid secretion (colloid droplets of Biondi, chromophobic vacuoles of Anderson, granules of Galeotti, of Lobenhofer, of Kinosita, basal colloid of Bensley, granules stainable by the neutral red of Uhlenhuth, etc.), but also on the possibility of their actual identification. When we started our work on the subject, the most recent reviews on the histophysiology of the thyroid gland, like those by Ponse (1938), Cowdry (1938), Bloom (1939), and Bargmann (1939), had reached the conclusion that secretion of the thyroid gland is invisible or that the intracellullar secretory processes could not be observed microscopically with certainty.

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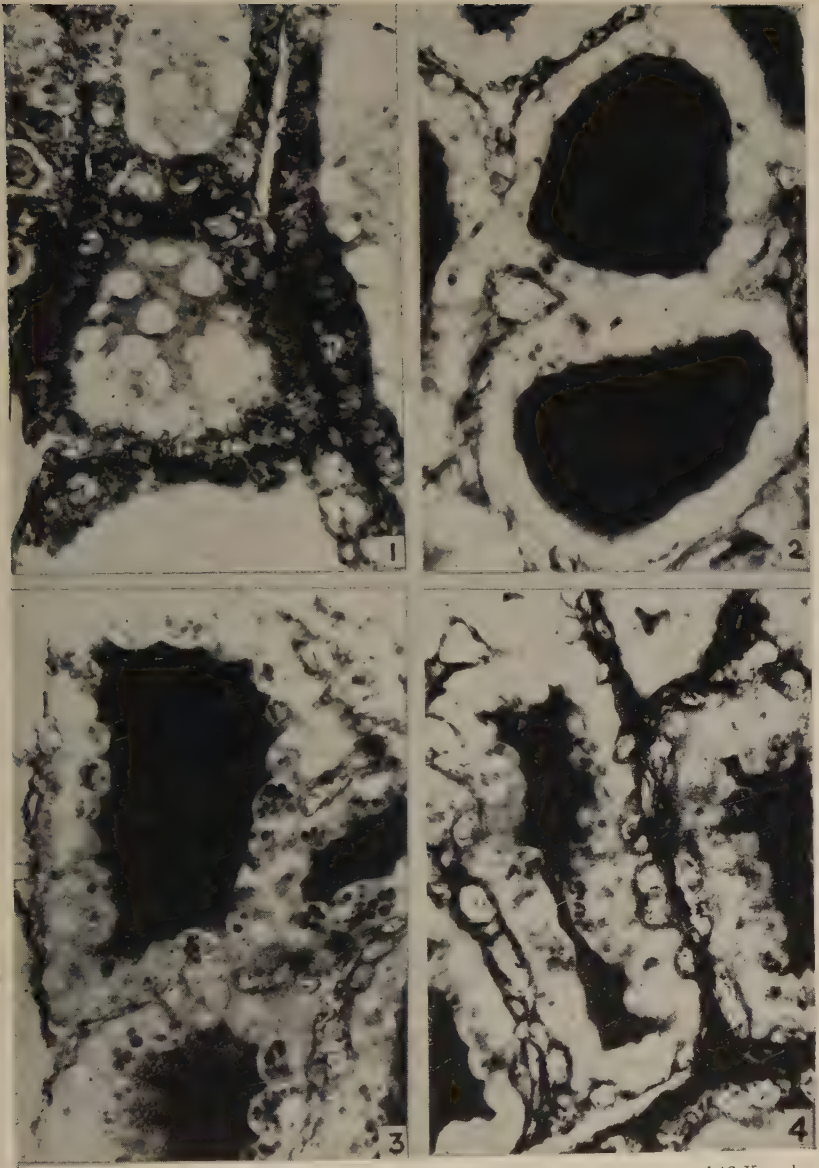
† Fellow of the Guggenheim Foundation.

A priori, it might be thought that this uncertainty was due to the lack of adequate technical methods for fixing and staining the colloid within the cell and, for this reason, in 1940, we used the freezing-drying method of Altmann-Gersh. This preserves best the different cellular components, particularly the proteins taking part in the secretion. This technique, followed by a simple staining method, reveals the products of secretion and reabsorption inside the cells, which we designated as intracellular colloid (De Robertis, 1941a), and we undertook a study of its changes in different physiological and pathological conditions. FIGURES 1 and 2 show the difference between the image produced by a chemical fixative like Regaud's fluid (FIGURE 1) and the freezing-drying technique (FIGURE 2). In the first figure, the follicular colloid shows many clear vacuoles, situated mainly at the periphery of the follicles, as is usual with common techniques. These vacuoles, described by all the authors since Anderson (1894), have been interpreted by some as chromophobic colloid being secreted (Anderson, 1894; Uhlenhuth, 1927; Charipper, 1926; etc.) and by others as vacuoles of reabsorption (Aron, 1930; Severinghaus, 1933) because they appear especially in activated glands. However, recently, several investigators have indicated that those vacuoles could be interpreted as artifacts, basing their supposition mainly on the action of different fixatives (Bucher, 1938) or on observations on living glands (Hartoch, 1933; Vonwiller and Vigodskaya, 1934; Williams, 1937–40; De Robertis, 1941b).

The freezing-drying method which was also applied by Gersh and Caspersson (1940) for observations with ultraviolet light showed definitively, in our hands (De Robertis, 1941a), that follicular vacuoles are artifacts produced by the fixatives while precipitating the gel which fills the thyroid follicle. Also, in strongly activated glands the follicular colloid is homogeneous (FIGURES 3 and 4).

Comparing both figures (1 and 2), it is seen also that, in the second case, the cells show the presence of colloid droplets inside the cytoplasm. The amount and disposition of the intracellular colloid varies with the species, with age, and with the season, and most markedly with functional (FIGURES 3 and 4) or pathological conditions in the gland. The colloid generally appears as droplets situated in the apical zone of the cell, but more rarely it is found in the basal part of the cell as large, pale-stained droplets similar to those described by Bensley (1916) as basal colloid. In a few cases, particularly in hyperactive glands, all the colloid droplets of a cell are at the base and the cell shows a complete inversion of polarity (FIGURE 6; De Robertis, 1941c). That the intracellular colloid described by us contains the thyroid hormone, seems to be apparent from a comparison of our results with those obtained by Gersh and Caspersson (1940) using the microspectrographic technique in ultraviolet light, with which they found specific absorption curves of organic iodine in vacuoles situated in the apical part of activated cells. We compared the photomicrographs obtained by the authors with the slides from the



FIGURES 1 to 4, photomicrographs of thyroid glands taken with 3 mm. objective and 12 X ocular.

FIGURE 1. Normal thyroid of a young rat. Regaud's fixation and Bensley's acid fuchsin-methyl green technique for mitochondria. Intrafollicular colloid shows many artificial vacuoles.

FIGURE 2. Normal thyroid of a young rat. Frozen-dried denatured stained with aniline blue-orange. Cuboidal epithelium with few colloid droplets. Homogeneous colloid.

FIGURE 3. Rat of the same litter three hours after the injection of TSH. Cells are higher and show many colloid droplets. Colloid is being released.

FIGURE 4. Rat of the same litter ten days after injections of TSH. High cylindrical cells with many colloid droplets. Colloid greatly released.

same material (given to us by Dr. Gersh) made with our staining technique. However, more direct evidence is lacking.

From these results, it is also clear that the chromophobic secretion considered by many authors, such as Anderson (1894), Uhlenhuth (1927), Baillif (1937), von Hagen (1938), etc., to be the true thyroid secretion, probably is an artifact or is due to an incapacity of common fixatives to preserve the content of those vacuoles.

When rats or guinea pigs are injected with TSH, within 15 minutes one can find a great increase in intracellular colloid. Analysis of this early activation in rats and guinea pigs shows (De Robertis, 1942, and unpublished results; FIGURE 5) that, in an initial period (with its maximum at 60 minutes), the cells are actively secreting toward the lumen. Colloid droplets which are formed near the nucleus move toward the apex, at the same time increasing in size, and are excreted into the follicular cavity, FIGURE 5*a*, *b*. It may be that the excretion of these droplets into the lumen is preceded by a diminution of the surface tension in some parts of the apical surface. At any rate, at these points the cytoplasm enclosing the colloid droplets bulges into the lumen, to be released

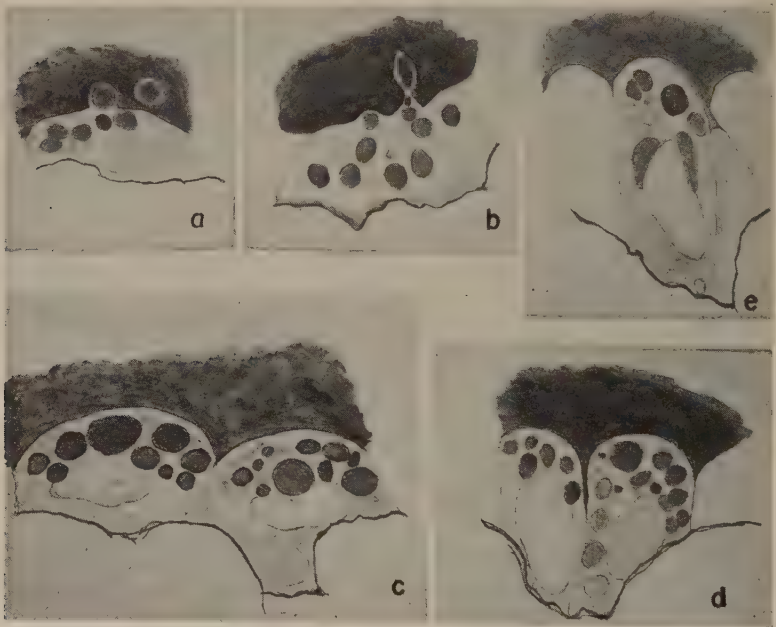


FIGURE 5. Drawings made with the help of the camera lucida of frozen-dried denatured sections of thyroid glands, stained with aniline blue-orange. 2150 X.

a, *b*. Thyroid cells of guinea pig 30 minutes after the injection of TSH. Phase of secretion toward the lumen. In *a*, a colloid droplet still surrounded by a rim of cytoplasm is seen in the lumen.

c. Phase of release six hours after the injection of TSH.

d, *e*. Phase of release six hours after the injection of TSH. Cells are higher, with convex apical border. Clear basal droplets of dilute colloid are seen.

finally by rupture of the pedicle. These processes suggest that thyroid cells produce an apocrine secretion, parts of their cytoplasm being excreted together with the secretion into the lumen.

After this phase, the cells appear to stop secreting toward the lumen, and to begin secretion toward the base, reabsorbing the colloid stored in the lumen. This assumption is further supported by the observations that (*1*) pale-staining, seemingly dilute colloid appears in the base of the cells; (*2*) in some thyroid cells, small colloid droplets are distributed in the basal half, resulting in a tendency toward a complete inversion of morphological polarity (FIGURE 6); and (*3*) intrafollicular colloid diminishes.

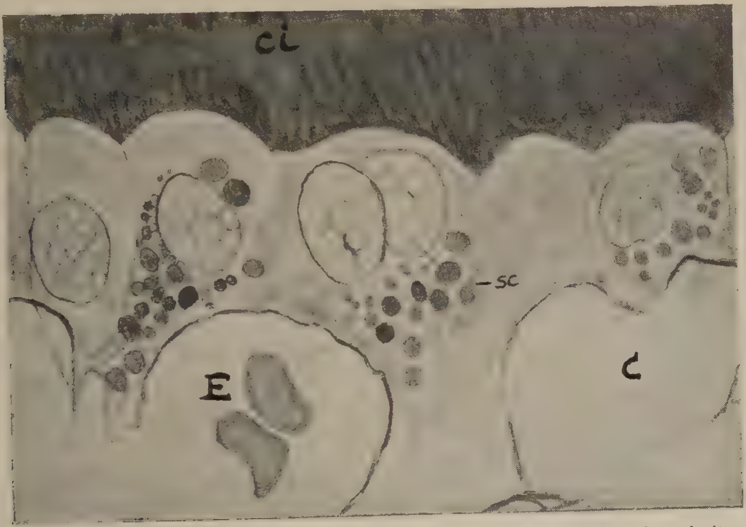


FIGURE 6. Thyroid cells of a rat injected 22 hours before with TSH. Inversion in the polarity of colloid droplets is shown. *ci*, intrafollicular colloid; *sc*, intracellular colloid; *E*, erythrocyte; *c*, capillary.

In the amphibian, the first phase of secretion toward the follicle could not be observed and a reabsorption with great increase of intracellular colloid took place (De Robertis and Del Conte, 1942).

The Cytological Method for the Assay of Thyrotropic Hormone. After the first experiments on rats, it became clear that the changes in amount and disposition of intracellular colloid were a very sensitive index of the functional activity of the gland and that these changes reflected in a sensitive manner the amount of circulating thyrotropic hormone. One species considered as insensitive to the TSH (Aron, 1932; Houssay *et al.*, 1932; Thurston, 1933), namely, the rat, reacted with a definite increase in intracellular colloid with 0.5 Junkmann-Schoeller unit (De Robertis, 1942). More recently, there was found a definite reaction with 0.1 (Grasso, 1946) and even with 0.01 of a unit (unpublished results). In a sensitive animal like the guinea pig, we found a definite increase of thyrotropic hormone with 0.001 and even with 0.0002 of a unit. Furthermore, the reaction seemed proportional to the dose so that a quantitative method was developed based on the relative amount of intracellular colloid (De Robertis and Del Conte, 1944). The technique consists in injecting the sample intravenously into the guinea pig, and in fixing the gland in liquid air 30 minutes later. Afterwards, the tissue is dried *in vacuo*, denatured and stained as usual, and the number of colloid droplets from 10 follicles is measured. A cytological coefficient was established as the ratio between the number of colloid droplets times 100 and the average diameter of the follicles:

$$\mathrm{Cc} = \frac{\text{No. of droplets} \times 100}{\begin{array}{c}\text{Medium follicular}\\ \text{diameter}\end{array}}$$

The cytological coefficient of the normal guinea pig thyroid is about 9.4, while in the rat it is a little higher, 13.04 (Grasso, 1946). As seen in FIGURE 7, the cytological coefficient increases with the dose and there exists an approximate proportion between this coefficient and the logarithm of the dose. Direct methods for the assay of TSH are generally based on changes in the weight of the thyroid gland and especially on histological changes such as the increase in height of thyroid cells. (For a discussion of the literature, see De Robertis and Del Conte, 1944.) This particular method is based on a cytological variation in the amount of intracellular colloid, and we find these changes highly specific. This cytological method greatly surpasses in sensitivity other techniques for the assay of TSH. In fact, Rawson and Salter (1940), using changes in

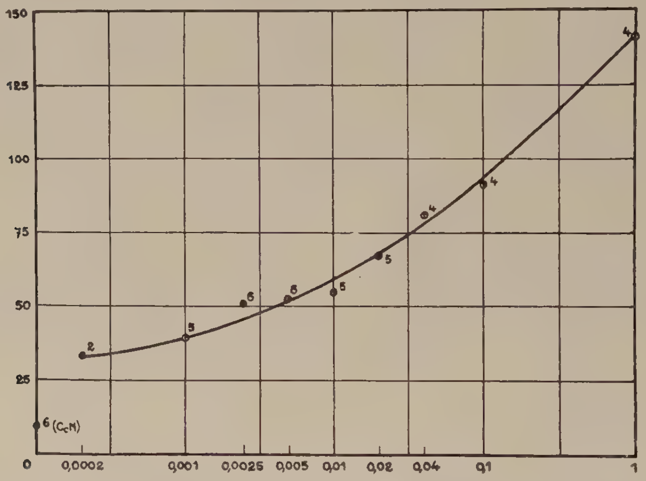


FIGURE 7. Curve plotting the relation between the cytological coefficient (0 to 150) and the doses of TSH 90.0002 to 1 Junkmann-Schoeller unit injected into the guinea pig expressed in logarithmic scale. Points indicate the means of the results for each dose and the number of animals. *CcN*, normal coefficient.

cell height in day-old chicks, could demonstrate only 1/8 of a J–S unit injected during 5 days. Galli-Mainini (1943), in the hypophysectomized toad, found the maximum of sensitivity with 1/10 of a unit. The direct method of D'Angelo Gordon and Charipper (1942) on frog tadpoles permits the determination of 1/320 of a unit, or, when indirect phenomena such as changes in metamorphosis (which are not completely specific) are used, a sensitivity of 1/1,280 of a unit can be attained. However, a great limitation for the use of this method is the small amount of fluid that can be injected into the tadpole (0.05 ml.), so that the TSH can be determined only in liquids where it is in rather high concentrations (1/16 unit per ml.).

Using the cytological method, if one injects 1 or 2 ml. of fluid into a guinea pig, one can demonstrate TSH in a concentration of 1/5,000 or 1/10,000 of a unit per ml. With this method, it is possible to demonstrate the presence of TSH in 2 ml. of normal human blood and to find great changes in amount with different pathological conditions. We believe that the specificity and extreme sensitivity of this method, when applied to human fluids, may permit clarification of the exact role of TSH in the pathology of the thyroid gland.*

The Oxidation-Reduction Potential and the Peroxidase System of the Thyroid Follicles. In 1941, we found that the pH of the follicular colloid determined by a microdissection technique did not change with the state of activity of the gland. We then became interested in the histochemical study of the oxidation-reduction potential as an expression of thyroid physiology. This interested us because, in a given cell or tissue, the level of the oxidation-reduction potential is determined by the activity of the enzymatic systems present, the O_2 pressure, and the nature and concentrations of the substrates (Stiehler and Flexner, 1938). It may also indicate how the oxidation-reduction energy can be utilized in the secretory mechanism. Even more interest arose when it was found that a series of substances such as thioureas and sulfonamides inhibit thyroid secretion, opening in this way a new field in thyroid physiology and a better approach to the study of the mechanism of the synthesis of the thyroid hormone.

The oxidation-reduction potential of the follicular colloid was determined in the thyroid of the rat by introducing various indicators into the lumen with a micropipette or by extracting the colloid from a single follicle and mixing it in the micropipette under the microscope with a small amount (about 2×10^{-6} ml.) of the indicator. The potential of the thyroid cells and of the colloid was studied by the supravital technique described by Friedenwald and Stiehler (1938; see De Robertis and Moura Gonçalvez, 1945). Results are shown in TABLES 1 and 2. It is seen that, in the normal rat, the potential of the cells is somewhere between +0.110 volts and +0.047 volts, while in the colloid it is between −0.167 and

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* Data on the assay of TSH in blood of human patients will soon be published. (De Robertis, E. P. 1948. J. Clin. Endocrinol.)

TABLE 1

OXIDO-REDUCTION POTENTIAL OF THE COLLOID DETERMINED BY MICRODISSECTION

Indicators	*Eo′ at pH 7*	*Normal rats*	*Activated rats*	*Action KCN in activated rats*	*Action thiourea in activated rats*
2–6 Dichlorophenol-indophenol	+0,217		+		
Thionin (Lauth's violet)	+0,100*	+	+		
Cresyl blue	+0,047	+	—		
Methylene blue	+0,011	+	—	+	+
Indig. tetrasulfonate	—0,046	+	—	+	+
Indig. trisulfonate	—0,081	+			
Nile blue	+0,122	+	—	+	+
Cresyl violet	—0,167	+	—	+	+
Phenosaphranine	—0,252	—	—	—	—
Neutral red	—0,330	—	—	—	—

* Eo′ at pH 6.0.

TABLE 2

OXIDO-REDUCTION POTENTIAL OF THE COLLOID DETERMINED BY SUPRAVITAL TECHNIQUE

Indicators	*Eo′ at pH 7*	*Normal rats*		*Activ. rats*		*Action KCN*		*Thiourea*		*Sulf.*	
		Cells	*Colloid*	*Cells*	*Colloid*	*Cells*	*Colloid*	*Cells*	*Colloid*	*C.*	*C.*
Thionin (Lauth's violet)	—0,110*	+	+	+	+	+	+	+	+	+	+
Cresyl blue	+0,047	—	+	—	—	+	+	+	+	—	+
Methylene blue	+0,011	—	+	—	—	+	+	+	+	—	+
Nile blue	+0,122	—	+	—	—	+	+	+	+	—	+
Cresyl violet	—0,167	—	+	—	—	+	+	+	+	—	+
Phenosaphranine	—0,252	—	—	—	—	—	—	—	—	—	—
Neutral red	—0,330	—	—	—	—	—	—	—	—	—	—

* Eo′ at pH 5.8.

—0.252 volts. When the gland is activated by the action of cold or by the injection of TSH, the potential of the colloid rises to the same level as that in the cells. Thiourea and KCN lower the potential of the cells and also that of the colloid in the case of activated glands. However, sulfonamide, another inhibitor of thyroid function, did not change the potential. These results led us to investigate the enzymatic systems that could be involved, particularly the oxidative systems. While this work was under way, there appeared the finding by Dempsey (1944) of the presence in the thyroid cells of a peroxidase activity which was inhibited by thiouracil. This observation was particularly important because peroxidases catalyze the liberation of iodine from iodides and their presence in the cells may be related to biological iodinations (Keston, 1944) and to the formation of thyroxine (Westerfeld and Lowe, 1942). The peroxidase activity was, therefore, studied with different techniques and in different conditions (De Robertis and Grasso, 1946). The results are very briefly summarized here. In the colloid extracted by microdissection (TABLE 3), both indophenoloxidase and peroxidase were negative in normal conditions, but the last was positive in glands activated by cold or by TSH. Both thiourea and KCN in concentration M 0.005 inhibited the reaction of the activated glands. With a supravital

TABLE 4

PEROXIDASE ACTIVITY OF CELLS AND COLLOID, DETERMINED WITH THE SUPRAVITAL TECHNIQUE

	Normal		*Activated*		*Action of KCN*		*Action of thiourea*		*Action of sulfanilamide*	
	Cells	*Colloid*	*Cells*	*Colloid*	*Cells*	*Colloid*	*Cells*	*Colloid*	*Cells*	*Colloid*
Peroxidase	+	—	+	+	—	—	—	—	+	—
Eo′ at pH 7 (volts, approx.)	+0.050	—0.200	+0.050	+0.050	—0.200	—0.200	—0.200	—0.200	+0.050	—0.200

TABLE 3

INDOPHENOLOXIDASE AND PEROXIDASE ACTIVITY IN THE COLLOID EXTRACTED BY MICRODISSECTION

	Normal rats	*Activated by cold*	*Activated by TSH*	*Action of thiourea on activated glands*	*Action of KCN on activated glands*
Indophenoloxidase	—	—	—		
Peroxidase	—	+	+	—	—
Eo′ at pH 7 (approx., volts)	−0.200	−0.050	−0.050	−0.200	−0.200

—, negative; +, positive. Eo′, oxidation-reduction potential. TSH, thyrotropic hormone.

technique, the same results were found for the colloid (TABLE 4). In normal and activated cells, peroxidase activity is positive and the reaction is inhibited by KCN and thiourea. However, sulfanilamide had no influence on the peroxidase activity. In both TABLES 3 and 4, results are correlated with the previous determinations of the oxidation-reduction potential.

In TABLE 5, it is shown that the histochemical reaction of peroxidase is completely inhibited with a concentration of thiourea of M 0.002, while with M 0.001 it is still very weak.

TABLE 5

ACTION OF THIOUREA AND SULFANILAMIDE IN FROZEN SECTIONS

	M 0.01	*M 0.005*	*M 0.0025*	*M 0.002*	*M 0.001*	*M 0.0001*
Control ++						
Thiourea	—	—	—	—	+	++
Sulfanilamide	+++	+++				

In another series of experiments, the action of thiourea on the liberation of iodine from iodides was tested. As seen in TABLE 6, the reaction was inhibited with M 0.001 thiourea. The same results were obtained with a potato extract containing a vegetable peroxidase. Opposite results were found after heating of the thyroid and potato extract or when using hemoglobin, brain and muscle extracts, which do not contain true

TABLE 6

ACTION OF THIOUREA ON THE LIBERATION OF IODINE FROM IODIDE

	Control	*M 0.03*	*M 0.01*	*M 0.005*	*M 0.0025*	*M 0.002*	*M 0.001*	*M 0.0001*
Thyroid extract	+		—	—	—	—	—	+
Id. heated at 120°C.	+		+	+	+	+	+	+
Potato extract	+		—	—	—	—	±	+
Id. heated at 120°C.	+		+	+	+	+	+	+
Hemoglobin	+	—	+	+	+	+	+	+
Id. heated	+		+	+	+	+	+	+
Brain extract	+		+	+	+	+	+	+
Muscle extract	+		—	+	+	+	+	+

peroxidase. Sulfathiazole also seems to stop the liberation of iodine, but probably by the formation of a new compound.

Time does not permit a general discussion of the literature on the mechanism of action of thyroid activators and inhibitors. We only want to point out that these results lead us to the conclusion that thiourea acts by inhibiting the peroxidase system, which in turn takes part in the liberation of iodine from iodides. Sulfonamides do not inhibit the peroxidase activity, but probably have a competitive action by which the liberated iodine combines with the sulfa drugs instead of with tyrosine.

(For more details in the discussion of this problem, see De Robertis and Grasso, 1946.)

The Proteolytic System and the Release of the Colloid from the Thyroid Gland. The fundamental problem of the release of the colloid stored in the follicles is that of the mechanism by which thyroglobulin is taken by the thyroid epithelium and transferred to the blood and lymphatic capillaries. Such a mechanism could be understood readily on the basis of the laws of permeability if very small molecules were involved. As Chambers and Zweifach (1946) state, "the upper limit of porosity of the cell membrane has never been claimed to be in excess of 342, the molecular volume of saccharose," and Wilbrant (1946) considers a molecular volume of 68,000 as the limiting porosity of the capillaries in the glomerular tufts of the kidney, where the transfer is probably through the cementing substance.

With the ultracentrifugation, it has been shown that thyroglobulin has a molecular volume of 675,000 (Heidelberger and Pedersen, 1936). It is really a macromolecule which, as such, cannot pass through the membranes of the cells.

In 1936, Salter and Lerman, by using total thyroid extracts, were able to synthesize iodoproteins which possessed activity similar to thyroglobulin and suggested the presence of an enzymatic mechanism which could act not only in the synthesis but also in the destruction of the thyroid protein. As Means (1937) stated, "The secretory activity of the thyroid gland could be compared with a reversible chemical reaction obeying the 'law of masses,' " so that, when the predominating reaction is in the direction of the synthesis, there would be an increase in the thyroid-protein (colloid), while when the process is reversed, thyroid-protein would be fragmented and more hormone would be released into the circulation.

In 1940, Gersh and Caspersson suggested the possibility that the follicular colloid could be digested by enzymatic action and the products absorbed by the cells. The same year (De Robertis, 1941b), we tried to confirm this hypothesis by testing the presence of proteases in the follicular colloid extracted by microdissection from single follicles. With this method, we were able to demonstrate the presence, in the colloid, of a proteolytic activity which varies in intensity according to the pH of the medium and the physiological activity of the gland. It was found that TSH, which produces hyperactivity and reabsorption of the colloid, increases the proteolytic activity, while iodine administered for a long period of time decreases it.

These facts led us to postulate the theory of the enzymatic reabsorption of the colloid, according to which the release of the secretion stored into the follicles takes place only after hydrolysis of thyroglobulin inside the follicular cavity. In the diagram (FIGURE 8), the mechanism is expressed graphically as it occurs in normal conditions and in the case of hyperfunction and hypofunction of the gland.

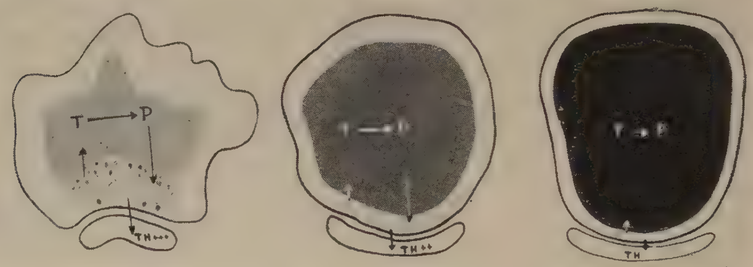

FIGURE 8. Diagram of the enzymatic mechanism of reabsorption of the colloid in normal conditions (middle), in a hyperactive follicle (left), and in a hypoactive one (left). Length and directions of the arrows indicate the intensity of processes of hydrolysis, reabsorption, apical and basal secretion. *T*, thyroglobulin; *P*, product of the hydrolysis of *T*; *TH*, circulant thyroid hormone. (The number of + indicates the amount of *TH* in each case.)

The experimental facts supporting this theory were fully confirmed by Dziemian (1943), using micromethods for proteases applied to the total gland. The author also found a lower P.A. after hypophysectomy.

These results strongly supported the assumption that a similar mechanism could exist in the human gland and that changes in this enzymatic activity might be related to diseases of the thyroid. In fact, the physiopathological mechanisms involved are essentially similar to those which occur in physiological or experimental conditions.

In toxic goiter, there is an increase in the release of the colloid, called by Holst (1927) and Harington (1933) "leakage" or "diarrhea" of the gland. This causes depletion of the colloidal stores. In simple goiter (at least in the phase of colloid goiter of Marine), there is an opposite phenomenon, namely, a diminution in the release, and a storage of colloid.

For these reasons, we carried out estimations of the enzymatic activity in a series of human thyroid glands representing normal and various pathological conditions (De Robertis and Nowinski, 1946a). It was found that, in cases of severe toxic goiters, there is an increase of P.A. to a point about 100 per cent above normal, while in simple colloid goiters the P.A. is about 30 per cent below the normal level (FIGURE 9). These results seem to indicate that the proteolytic system has an important role in the physiopathology of thyrotoxicosis and simple colloid goiter.

In cases of mild toxic goiters (starting with a B.M.R. of —40 per cent) which reached a complete relief of the symptoms with the iodine treatment, the P.A. is not only lower than that of severe toxic goiters but is even below the normal level (—26.2 per cent). It is interesting that, in these cases, the gland showed big follicles filled with colloid, as in simple goiter.

This fact, together with the diminution of P.A. found by us (De Robertis, 1941b) and by Dziemian (1943) in rats treated with iodide, may be interpreted on the basis of the enzymatic reabsorption of the colloid and suggest that the action of iodine in thyrotoxicosis may be attributed to the influence on the proteolytic enzyme system of the thyroid gland. The mechanism of iodine action in this inhibition may possibly be due to a direct iodinization of the proteolytic enzyme. Support of this idea is found in the paper by Herriott (1936) who, by iodinizing pepsin *in vitro*, observed a progressive inhibition in P.A. due to the fixation of iodine in the tyrosine molecule.

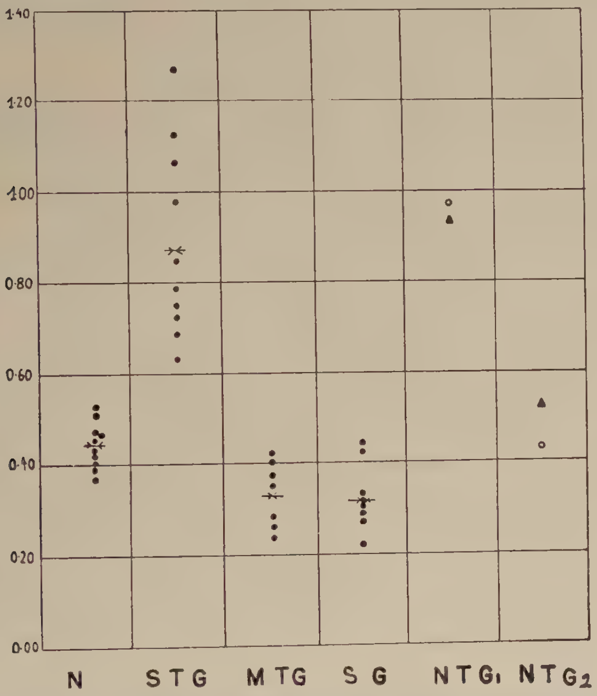

FIGURE 9. Diagram expressing the proteolytic activity of human thyroid tissue in normal glands (*N*), severe toxic goiter (*S.T.G.*), mild toxic goiter (*M.T.G.*), simple goiter (*S.G.*), nodular toxic goiter, toxic adenoma (*N.T.G.*$_1$), and the rest of the gland (*N.T.G.*$_2$). Proteolysis is measured by the mg. of tyrosine and tryptophane set free during the digestion of edestin by 100 mg. of tissue;→ ←means.

In order to check the direct action of iodine upon the proteolytic enzyme of the thyroid, we carried out a series of experiments in which the glycerol extracts of the gland were iodinized *in vitro* with Herriott's method. We found a strong inhibition of the P.A. (De Robertis and Nowinski, 1946b). However, our interpretation of the mechanism of the therapeutic action of iodine does not exclude the existence of other processes such as the action on TSH recently supported by Rawson and colleagues (1945). It is obvious that, in the case of a process so important physiologically as the regulation of hormone production, the cooperation of several systems must be involved.

Summary

Our knowledge of secretory processes in the thyroid gland was rather confused until 1939 because of the lack of adequate methods for preserving the products of secretion and reabsorption inside the cells. In 1940, the use of the freezing-drying technique enabled us to demonstrate the intracellular colloid. It was also found that vacuoles in the colloid and the so-called chromophobic secretion were artifacts.

Activation of the gland by TSH produces a great increase of intracellular colloid. This is noticeable as early as fifteen minutes after administration of the hormone. At first, the cells secrete toward the follicle, producing a kind of apocrine secretion which is very evident when the cells are strongly activated. Afterwards, apical secretion stops and release of the colloid through the cells is observed. In some cases, there is also an inversion of cell polarity. This increase of intracellular colloid is the most sensitive method for detecting TSH. In the guinea pig, the injection of 0.0002 unit of TSH produces a definite increase of the number of colloid droplets and the reaction is proportional to the dose, at least up to one unit of TSH. A cytological coefficient was established, based on the number of colloid droplets per follicle, and on this basis a method for the assay of TSH was developed.

This method is much more sensitive than others utilized for the assay of TSH, and permits one to demonstrate its presence in 2 ml. of human blood. Thus, it can be widely applied to determine the role of this hormone in different pathological conditions. The study of the oxido-reduction potential of the thyroid follicle shows that it is intimately related to the activity of the gland. In normal glands, the Eo′ of the cells is about +0.050 volts and the colloid—0.200 volts. During activation, the Eo′ of the colloid increases to +0.050. KNC and thiourea lower the Eo′ of cells and colloid to −0.200, but sulfa drugs do not change the potential. These results were related to changes in peroxidase activity under similar conditions. The conclusion is drawn that thiourea probably acts by inhibiting the peroxidase system which takes part in the liberation of iodine from iodides, while sulfonamides do not inhibit peroxidase activity and probably have a competitive action toward the essential metabolite.

The release of the colloid through the cells cannot be explained by laws of permeability because of the large size of the thyroglobulin

molecule. Hence, it was suggested that an enzymatic mechanism might be involved. In 1941, we found in colloid extracted from single follicles a proteolytic activity which increased with activation of the gland and decreased under opposite conditions. These observations led to the postulation of an enzymatic theory for the reabsorption of the follicular colloid.

Similar results were found in normal and pathological human thyroids. In severe toxic goiters, there is an increase of P.A. of about 100 per cent above normal. In simple colloid goiter, it is diminished to about —30 per cent. These results seem to indicate that the proteolytic system may have an important role in the physiopathology of these diseases.

Results on mild toxic goiters treated with iodine, together with previous experimental results, lead to the conclusion that probably the therapeutic action of iodine may be attributed to its influence upon the proteolytic system of the thyroid gland.

Experiments of iodinization *in vitro* show that this action is possibly due to a direct iodinization of the proteolytic enzyme.

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Discussion of the Paper

DR. C. P. LEBLOND (*McGill University, Montreal, Canada*):

The statement that the vacuoles and staining reactions of the colloid are artifacts should be qualified. It is true that they actually are artifacts since, after osmic-acid or freezing-drying fixation, the colloid shows no vacuoles and does not stain differentially with trichrome stains. However, in sections fixed with the usual fixatives, both the vacuoles and the staining reactions of the colloid have a definite meaning, since they vary considerably according to the physiological condition of the gland. The vacuoles, for instance, which are absent in the living animal and occur during the process of fixation, are most numerous in stimulated thyroids, but rare in resting glands. Similarly, the colloid is predominantly basophilic in activated glands and acidophilic in resting glands. Therefore, it may be concluded that the presence of vacuoles and basophilia in the colloid corresponds to properties of the colloid usually found in activated glands.

Furthermore, the behavior of the basophilic and acidophilic colloid toward radio-iodine is quite different. For instance, 24 hours after administration of a small dose of radio-iodine, the acidophilic follicles contain much more radio-iodine than the basophilic ones, contrary to what is found earlier after injection. This finding was interpreted as indicating that the turnover of iodine is more rapid in the basophilic follicles than in the acidophilic ones.

If colloid is smeared from a sliced thyroid (dog) onto a glass slide, this colloid may be fixed and stained and will appear either basophilic or acidophilic or show a variegated appearance, according to the state of the gland. If, in these conditions, acidophilic colloid is treated with trypsine, it will rapidly be transformed into basophilic colloid. From this experiment, it may be suggested that the basophilia is somehow connected with the presence of proteolytic enzymes, since Dr. De Robertis showed that the activated (predominantly basophilic) thyroids do contain an increased amount of proteolytic enzyme.

Dr. Victor M. Trikojus (*University of Melbourne, Melbourne, Australia*):

Reference was made to the work of Wright and Trikojus,[1] who demonstrated that iodine inactivation of the thyrotrophic hormone, presumably by oxidation, resulted *in vitro* with molecular iodine under physiological conditions as regards pH and temperature. (Similar findings have been recorded by Rawson and colleagues.) As the thyrotrophic hormone is known to exert its effect intra-thyroidally, the possibility of the favorable influence of iodine in Graves' disease being associated with inactivation of the thyrotrophic hormone should not be overlooked.

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Dr. E. D. Goldsmith (*New York University, New York, N. Y.*):

With reference to Dr. De Robertis's observation that sulfanilamide does not inactivate peroxidase, it is of interest to recall that we had reported (Growth 9, 1945) that sulfadiazine and para-aminobenzoic acid exerted no action on the effects of thyrotrophic hormone in *Rana pipiens* larvae. Two mg. of sulfadiazine and 1 mg. of para-aminobenzoic acid in 0.05 cc. distilled water were injected pleuro-peritoneally on alternate days. Similar treatment with 1 mg. of thiourea did antagonize the effects of thyrotrophic hormone in amphibian development. Similarly, in the chick, sulfonamides have been found ineffective as an antithyroid agent. However, Dr. MacKenzie can tell us more about that.

Dr. C. G. MacKenzie (*Department of Biochemistry, Cornell University Medical College, New York, N. Y.*):

I wonder if an alternative explanation of the effects of thiourea and sulfanilamide on thyroid peroxidase is not permissible. Recently, Randall has shown that thioureas, far from inhibiting horse-radish peroxidase, are themselves substrates for the peroxide-peroxidase system. If the postulated thyroid peroxidase is similar to this one, it seems that thioureas, rather than inhibiting it, may compete with iodide for the enzyme-peroxide complex, and also reduce any oxidized iodine as fast as it is formed.

With respect to the sulfonamides, Lipmann reported that they were not oxidized by horse-radish peroxidase plus peroxide, but that they inhibited the oxidation of PABA, and that H_2O_2 did not disappear in their presence. This suggests that they inhibit the enzyme in a non-competitive fashion.

Dr. E. De Robertis:

Although other interpretations of the mechanism of inhibitors can be supported, the one favored by us seems to explain better our experimental results.

The inhibiting effect of thiourea on vegetable-peroxidase is a well known fact as stated by Sumner and Somers.[1] In our experiments the histochemical reaction of peroxidase with benzidine was inhibited with 0.001 M thiourea, while the unspecific reaction shown by hemoglobin was "inhibited" only with 0.03 M. Furthermore, in our experiments on the liberation of iodine from iodides, 0.03 M thiourea was needed to prevent the formation of free iodine in a peroxide-iodide system, while 0.001 M thiourea stopped the reaction when peroxidase was also present. These facts can be explained by the extreme rapidity with which peroxide and peroxidase unite together to form a relatively tight complex.[2]

Mann and Keilin[3] have shown that sulfonamide does not inhibit perox idase even at 0.01 M concentration, and Wood[4] demonstrated that sulfonamide may act in a competitive way on the oxidation of *p*-aminobenzoic acid with peroxidase. It is also known that, when catecol is oxidized by tyrosinase, an *o*-quinone is formed which may react with sulfanilamide to form a red compound. The facts observed by us—that sulfa-drugs do not lower the potential redox (as does thiourea), that they do not inhibit the histochemical reaction with benzidine, that sulfathiazole with iodine forms a yellow compound, and that sulfanilamide injected into the animal produces a reddish tinge in the thyroid gland—can be better explained by the supposition that these compounds act in a competitive way with the "essential metabolite."

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THE CHEMICAL CYTOLOGY OF THE THYROID GLAND

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IN recent years, rapid strides have been made in applying procedures for chemically characterizing cells and cellular inclusions. A potentially great advantage resides in these procedures in that they reveal the location of metabolically active substances in tissues and cells. Experience has shown that many such substances are sharply segregated, so that their local or effective concentrations may be many times greater than their apparent concentrations as judged by analyses of tissue homogenates.

Although there are many inherent advantages in a combined chemical and cytological approach, not enough information is presently available on which to base far-reaching generalizations. Practical difficulties, the non-specific or semi-specific character of many of the methods, and the relative newness of the field all contribute to a lack of sufficient trustworthy data. Consequently, at the present moment, one can only tentatively make correlations and draw conclusions which may be useful in constructing working hypotheses, but which cannot be regarded as sufficiently well established for a more solid theoretical structure.

General Histophysiological Considerations. The parenchymatous portion of the thyroid is derived from a median downgrowth of entodermal cells from the tongue which proliferate to form cords or sheets. These cells become separated into small masses, in the center of which a lumen appears. Thus, the mature thyroid gland consists of a single layer of epithelial cells arranged in follicles which contain a transparent gelatinous colloid. The follicular cells may be columnar, cuboidal or flat, depending upon whether small, moderate or large amounts of colloid are present. Occasional cords or groups of cells without lumens occur between the follicles and constitute the interfollicular cells.

The follicular cells exhibit well-defined mitochondria, Golgi nets, and intracellular vacuoles. Both mitochondrial and Golgi material increase in states of hyperactivity. The Golgi apparatus normally lies above the centrally located, rounded nucleus. It has been claimed that this apparatus reverses its position and becomes infranuclear when the follicle is activated, but modern work has not confirmed this notion. Lipoid-containing vacuoles are present in the apices of the follicular cells. During active states, the amount of intracellular lipoid increases. Other vacuoles, containing a colorless fluid,[44] the tinctorial properties of which are

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* This work was done in part under a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

similar to those of the intrafollicular colloid,[5] also occur within the cells. Gersh and Caspersson[15] have observed more recently that ultra violet light is absorbed comparably by these vacuoles and by the colloid.

The follicular cells rest upon a delicate network of argyrophilic reticular tissue (FIGURES 6 and 7). There is no well-defined basement membrane, although a thin homogenous line is stained with the azan procedure. The follicular cells may easily become detached and float free into the colloid. Occasional, usually solitary, mast cells are scattered in the reticular framework of the gland in some animals. A rich vascular plexus permeates the stroma of the gland, surrounding the individual follicles by a network of anastomosing capillaries. These capillaries frequently indent the basal cytoplasm of the follicular cells so that their courses appear to be intraepithelial.

The basic pattern of the thyroid gland is similar in all vertebrate animals. The relative proportions of cells and colloid appear to constitute the principal variations among the various species. Thus, the rhesus monkey has large follicles plentifully filled with thick, intensely chromophilic colloid and an excessively thin and flat epithelial layer, whereas in the mouse and rat the follicular cells are relatively tall and the colloid is correspondingly meager and thin in consistency. A well-developed zonation occurs within the thyroid of these last two species. The peripherally located follicles are among the largest in the gland and contain dense colloid which stains blue with the eosin methylene blue method and red with the triacid azan. The central portion of the thyroid, on the contrary, has small follicles, the colloid of which is scanty and stains weakly with basic dyes, and with the blue, rather than the red, component of the azan stain. The cells of these central follicles are high cuboidal or columnar in shape. Local variations in appearance also characterize the human thyroid. Microfollicular and macrofollicular areas are present, and occasionally regions or cushions of tall columnar cells occur within follicles with otherwise flattened epithelium.

Alterations in the microscopical appearance of the thyroid gland may be induced in animals by appropriate experimental procedures. Hypophysectomy in the rat is followed by shrinkage in the total size of the gland. Individual follicles, however, contain increased amounts of colloid and the follicular cells become excessively flattened.[39] On the other hand, administration of pituitary extracts containing the thyroid-stimulating principle, thyrotropin, depletes the intrafollicular colloid and increases the height of the follicular cells. Similarly, Cramer[6] has shown that exposure to a hot environment causes an increased deposition of colloid, whereas cold induces discharge of the colloid into the circulation. Corresponding and concomitant alterations in the Golgi apparatus, mitochondria, and cell-height attend these experimental procedures. These effects of environmental temperature apparently are mediated through the anterior pituitary gland, since the expected changes do not occur in hypophysectomized rats or in rats in which the pituitary stalk has been transected.[43] These findings have been interpreted to mean that the thyroid actively secretes its stored colloid under the stimulus of pituitary thyrotropin and that colloid is stored when the thyrotropin is decreased. Such an interpretation is in good accord with pathological observations which have correlated hyperthyroid states with depleted colloid stores and hyperplastic epithelium.

Recently, drugs which profoundly modify the activity and appearance of the thyroid have been discovered.[1-3, 34, 37] These drugs, for example thiouracil, prevent the synthesis of hormone by the thyroid gland but do not affect the peripheral action of thyroid hormone, nor do they interfere with the pituitary-induced hyperplasia of the gland. These inferences are drawn from Astwood's[3] and the MacKenzies'[34] experiments, which establish the facts that thiouracil causes a profound hyperplasia and loss of colloid in normal but not in hypophysectomized rats, and that the hyperplastic effects of the drug may be nullified by concomitant administration of thyroid hormone. Indeed, Dempsey and Astwood[8] found the latter effect to be so striking that it provides a method sensitive enough to detect altered rates of thyroid secretion in rats maintained in hot and cold environments. Mixner, Reineke, and Turner[36] have obtained similar results in different breeds of fowl.

To summarize these various histophysiological data, it may be said that morphological changes occur which characterize states of increased or decreased thyroidal activity. Differences in the morphological appearances of the thyroids of various animals suggest that some, such as the monkey, have low rates of activity, whereas others, for example the rat, have high rates. Experimental procedures, involving subjection of rats to hot and cold environments, hypophysectomy and replacement therapy with thyrotropin, and treatment with antithyroid drugs and with thyroid hormone, permit the regulation of thyroid activity at any desired level. The thyroid gland, therefore, is an admirable organ for chemocytological analysis, since its histophysiological variations are well understood and its physiological activity may be regulated easily.

Basophilic Substances of the Thyroid Gland. In eosin-methylene blue preparations of the thyroid gland, various objects exhibit basophilic properties. The follicular cells contain basophilic nuclei, and, in addition, a fine stippling of basophilic substance occurs in the cytoplasm. The thick, inspissated colloid of large follicles stains deeply blue, whereas the thinner colloid of more active follicles becomes faintly or moderately colored (FIGURES 1 and 3). Finally, the moderately abundant mast cells of the thyroid stroma contain granules which stain intensely with methylene blue.

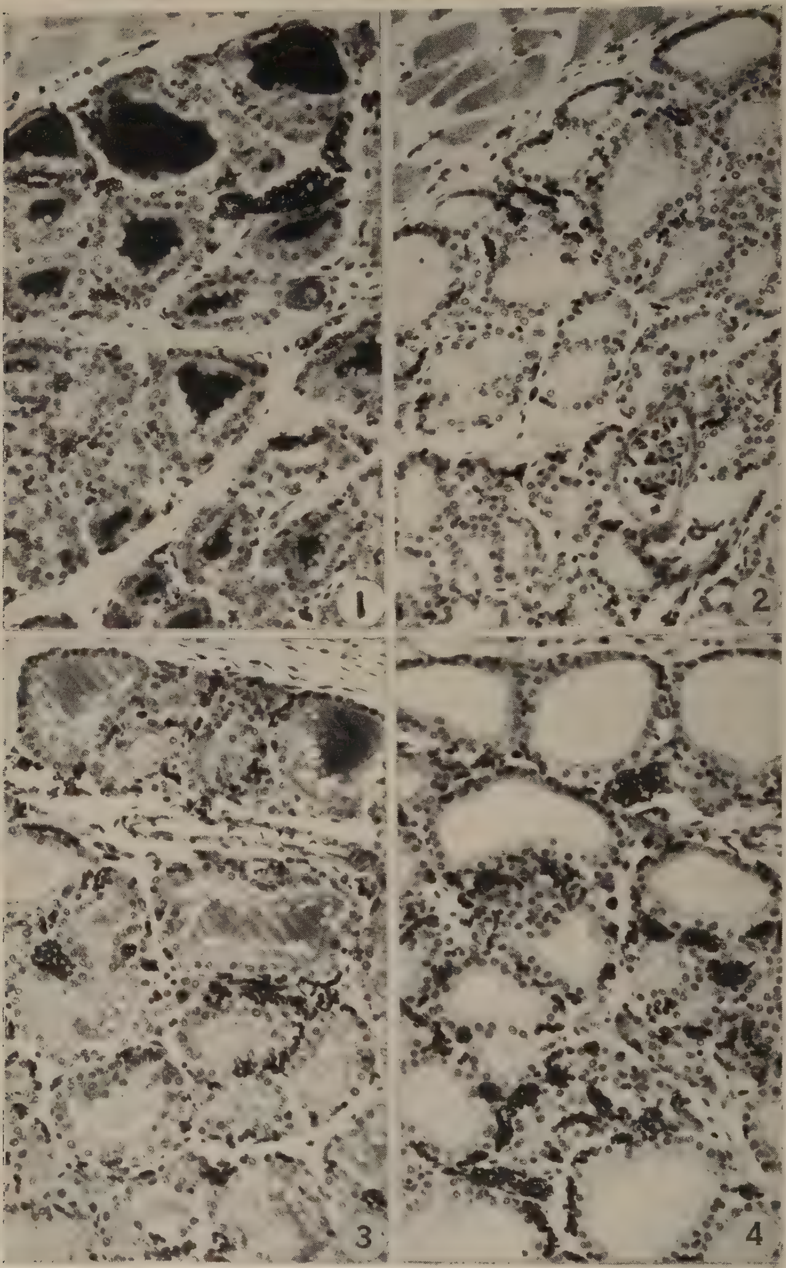
The nuclei of the follicular and stromal cells stain positively with the Feulgen procedure. The Feulgen reaction is usually regarded as specific for the nuclear nucleoproteins which contain desoxyribose.[40] The nuclei of peripheral, inactive follicles in the rat thyroid stain slightly more deeply than do those of the central, active follicles.[9] Similarly, stimulation of the thyroid by exposure of rats to cold and by the administration of thiouracil causes a slight diminution in the Feulgen reaction of the nuclei of the follicles. Presumably, therefore, states of active secretion lead to a slight decrease in the nuclear Feulgen reaction.

The cytoplasm of the follicular cells, the intrafollicular colloid, and the mast cell granules are all negative when tested by the Feulgen procedure. The intrafollicular colloid frequently takes on a purplish tint in Feulgen preparations. This does not represent a positive reaction, however, since a similar tinge occurs when thyroids are stained by the Bauer[4] procedure for glycogen and by the plasmal reaction of Feulgen and Voit.[13] The essential difference in these various reactions concerns the kind of hydrolysis to which the tissue is subjected prior to treatment with Schiff's leucofuchsin reagent. The specificity of the Feulgen reaction derives from the character of the initial hydrolysis in warm hydrochloric acid; consequently, the purplish tinge referred to above cannot be ascribed to the presence of desoxyribonucleoprotein in the colloid. The results obtained with the Feulgen reaction, therefore, indicate that the nuclear basophilia of the thyroid cells can be satisfactorily accounted for by their content of desoxyribonucleoprotein, but that the basophilic properties of the cytoplasm, colloid, and mast-cell granules must find some other explanation.

Cytoplasmic, or ribose-containing, nucleoproteins may be characterized in other locations by their lability to digestion by ribonuclease.[10] After incubation of sections of thyroid gland in solutions of crystalline enzyme, the follicular cytoplasm and the colloid both fail to stain with methylene blue (FIGURES 2 and 4). On the other hand, the chromatin of the nuclei and the granules of the mast cells are unaffected by treatment with the enzyme.

The destruction of basophilia in the cytoplasm and colloid by digestion in ribonuclease strongly suggests that these regions contain appreciable concentrations of ribonucleoproteins. However, such a conclusion is open to the criticism that ribonuclease may be non-specific in its action and may, in addition to depolymerizing ribonucleoproteins, also cause changes in other proteins of fixed tissues. Fortunately, this possibility may be tested by a second method for characterizing nucleoproteins.

The staining reactions of proteins under controlled physicochemical conditions may serve to characterize certain substances. Solutions of 5×10^{-4} molar methylene blue were adjusted to various pH values with acetate or phosphate buffers having an ionic strength of 0.01. Sections of Zenker-fixed thyroids were placed in 1500 cc. of these solutions and stained at 25± 0.1° C. for 24 hours, during which time equilibrium was attained. The sections were rapidly dehydrated and covered as permanent preparations. The intensity of staining of the various tissue elements was determined by projecting the image of the section upon the



FIGURES 1–4 (*See opposite page*)

ground-glass viewing plate of a photomicrographic camera. The amount of light transmitted through a given portion of the tissue was measured with a Model 512 Photovolt light meter, the search unit of which was fitted with an extension piece having an aperture $\frac{1}{8}$ inch in diameter. The light absorbed was calculated as per cent and plotted against pH to form a graph relating staining affinity to the acidity of the staining solution (FIGURE 5).

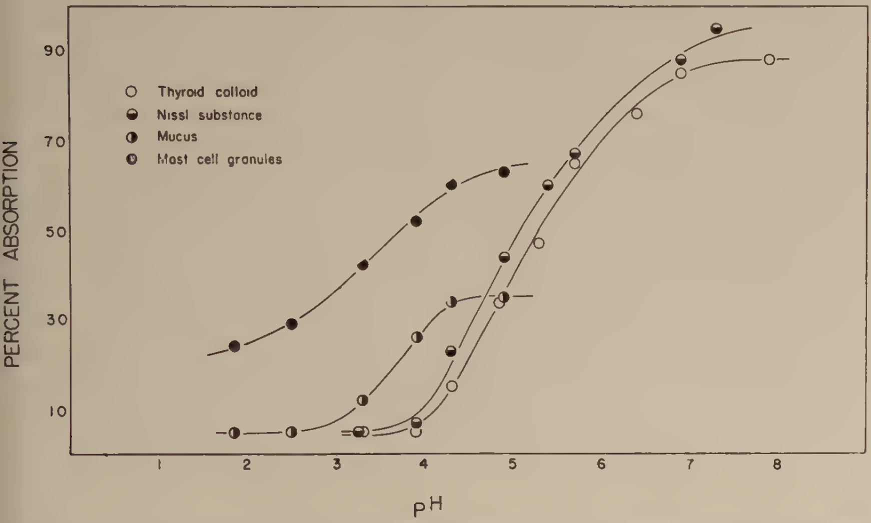


FIGURE 5. A comparison of the binding capacity for methylene blue of several basophilic substances over a range of pH. Nissl substance, a known ribonucleoprotein, and thyroid colloid fail to stain below pH 4.0 and attain a maximal uptake of dye at about pH 7.0. On the other hand, mucus and the granules of mast cells continue to stain in solutions considerably more acid than pH 4.0. This behavior presumably indicates more strongly dissociating acid groups in the latter two substances.

Curves constructed for thyroid colloid indicate that staining affinity increases sharply between pH 4.0 and pH 7.0. Below pH 4.0, therefore, the negative charge of the colloid is suppressed by the acidity of the staining solution, whereas at and above pH 7.0 the negative charge has attained its maximum. For comparison, a similar curve constructed for

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FIGURES 1–4 (*See opposite page*)

The effect of ribonuclease upon the basophilia of the thyroid gland. The four sections on this plate were prepared from Zenker-fixed thyroids and were stained identically by the eosin-methylene blue procedure. The photographs were exposed, developed, and printed for identical times. All sections X 200.

FIGURE 1. Thyroid gland from normal control rat. The peripheral follicles (upper part of figure) contain intensely basophilic colloid.

FIGURE 2. Section adjacent to that presented in FIGURE 1, and treated identically except for digestion in a solution of crystalline ribonuclease. The basophilic reaction of both colloid and cytoplasm is greatly depressed.

FIGURE 3. Thyroid gland from a rat maintained in a cold room. The basophilia of the colloid is reduced as compared with that of the control rat shown in FIGURE 1.

FIGURE 4. Section adjacent to that presented in FIGURE 3, and treated identically after digestion in ribonuclease. The basophilia of the colloid has been abolished.

Nissl bodies from nerve cells in which the presence of nucleoprotein has been well substantiated indicates a similar suppression of staining below pH 4.0 and a maximum uptake of basic dye at about pH 7.0. Consequently, the position and character of the curve relating pH to staining capacity provide evidence that nucleoprotein is a constituent of thyroid colloid. This conclusion fortifies the similar one derived from digestion of sections with ribonuclease. It appears, therefore, that the basophilic properties of the colloid of the follicular cytoplasm may be accounted for by their content of ribonucleoproteins.

The remaining basophilic element of the thyroid, namely, the mast cell, is colored metachromatically with methylene blue and also by toluidin blue after Holmgren and Wilander's[29] procedure involving fixation in basic lead acetate. According to these authors, such metachromatic staining indicates the presence of sulfate-containing mucopolysaccharide complexes. The nuclei and cytoplasm of the follicular cells and the intrafollicular colloid, on the other hand, do not stain metachromatically. The metachromatic basophilia of mast-cell granules is unaffected by digestion in ribonuclease. The signature of these granules, when obtained by measuring their affinity for methylene blue at various pH values, is strikingly different from those of colloid and nuclei (FIGURE 5). The granules continue to stain appreciably in solutions of pH 1.8, indicating much stronger dissociation of their negative charges than is the case for those of nucleoproteins, a fact consonant with the thought that half-esters of sulfuric acid are an integral part of their composition. These observations indicate that the granules of mast cells do not exhibit the expected reactions of nucleoproteins but behave in a fashion characteristic of sulfate-containing mucopolysaccharide substances.

In summary of the above, the basophilic properties of the various thyroid components may be satisfactorily correlated with their other chemical characteristics. The nuclei apparently contain desoxyribonucleoproteins. The cytoplasm of the follicular cells and the intrafollicular colloid react like ribonucleoproteins. The granules of mast cells exhibit behavior characteristic of mucopolysaccharides.

Autofluorescence of the Thyroid. The fluorescence of tissues, both in their natural state and after reaction with fluorochromes, has been exploited only very slightly by histologists. The thyroid gland is no exception to this statement, there being few studies available on thyroidal fluorescence. Hartoch[27, 28] has studied the fluorescence of the thyroid of the living rat with fluorochromes. Hamperl[26] has investigated the autofluorescence of human thyroids, and Grafflin has published a series of observations on the deer,[18, 19, 22] the gorilla,[20] the baboon,[21, 24] the chimpanzee,[24] and the elephant.[23] Dempsey[7] investigated the autofluorescence of the rat thyroid after various fixatives and physiological procedures.

The connective tissue framework of the thyroid is fluorescent in all of the species studied. There is good agreement among the various authors as to its bluish or bluish-white color. In general, the denser capsule fluoresces more intensely than do the more delicate strands of interfollicular tissue. Occasional pigment bodies are scattered among the stromal cells in many species. These pigmented areas exhibit a reddish or brownish-yellow fluorescence, and have been designated as "wear and tear" pigment (*Abnutzungspigment*) by Hamperl[26] and Grafflin.[18]

The fluorescence of colloid varies greatly in individual follicles and in diverse animals. Moreover, according to Dempsey,[7] the previous chemical treatment involved in the kind of fixation also determines the kind of fluorescence observed. After formaldehyde, which Hamperl[26] and Grafflin[18] have adopted as the fixative of choice, the majority of the follicles contain colloid which fluoresces weakly or not at all. Occasional follicles, increasing in number with age and correlated with increased density of the colloid, fluoresce more intensely with a bluish or bluish-white color, sometimes with a greenish component. This fluorescent colloid may be dispersed evenly in the follicle or may occur as irregular granules and masses within a matrix of weakly fluorescent colloid. The distribution of this highly fluorescent colloid is similar, in several species, to the distribution of dense, basophilic colloid which also exists variably as granules, as masses, or homogeneously throughout individual follicles. There seems good reason, therefore, to equate the strong fluorescence of these areas with the more dense aggregation of the colloid in some follicles.[18]

In addition to the fluorescence of the colloid itself, Grafflin[22] has described crystals which fluoresce a yellowish color and which occur occasionally in the follicles of the deer thyroid. The nature of these crystals is undetermined.

The fundamental fluorescent color of the follicular cells appears to be an ill-defined bluish or bluish-white. The intensity varies considerably in different species. In the baboon the cells are practically non-fluorescent,[21] whereas in the rat the intensity is high and of a whitish quality.[7] Most animals show, in addition, a yellowish fluorescence of a granular character, concentrated for the most part in the apical portions of the follicular cells. The distribution of these yellowish granules corresponds to that of yellow pigment granules, which may frequently be observed in the thyroid epithelium. It would appear that young animals contain smaller numbers of these yellow granules than do older animals. This and other considerations have led Hamperl and Grafflin to the conclusion that they represent intracellular "wear and tear" pigment.

A brownish pigment which is preserved in formaldehyde but is destroyed by weak solutions of KOH has been observed by Grafflin[20] in both the parenchymatous and the supporting tissues of the thyroid of the gorilla and baboon. This pigment fluoresces a well-defined red color, a circumstance which suggests its possible porphyrin nature.

Although the fluorescence of the colloid in the majority of the follicles is slight after fixation of the thyroid in formaldehyde, fluorescence is greatly enhanced after fixation in alcohol.[7] Moreover, the intensity of fluorescence varies considerably in different physiological states. The stored colloid of inactive follicles fluoresces intensely, whereas weaker reactions occur in follicles from animals maintained in cold environments. Weak fluorescence also characterizes the small residuum of colloid in the thyroids of animals to which thiouracil had been administered. The reconstituted colloid which accumulates after hypophysectomy is also deficient in fluorescence, suggesting that the fluorescence may be related to the hormone concentration in the gland, since the colloid produced after these experimental procedures is deficient in iodine.

Iron Reactions of the Thyroid. Iron may be demonstrated in tissue sections by two principal procedures, namely, by Turnbull blue or Prussian blue reactions, in which iron enters into reactions with a colored end-product, and by the observation of a yellow, orange or red ash in micro-incinerated specimens.[38] The color reactions are precise and satisfactory, but unfortunately only part of the total iron contained in the tissue is revealed by them. Microincineration causes drastic bubbling of the tissue with, consequently, the possibility of rearrangement of the various components. The cytological location of iron as revealed by this method is therefore open to question. Despite this fault, the total quantity of iron, metallic or organic and masked or unmasked, is unquestionably placed in evidence.

Iron stainable by the Prussian blue or Turnbull blue procedures is usually absent in the thyroid. However, occasionally, a single follicle or an area composed of a few follicles with their adjacent stroma will be observed to stain. In these rare areas, the stain may be barely perceptible, or, upon occasion, be fairly intense. Macrophages containing hemosiderin are usually present in the stroma contiguous to these areas. There seems little reason to doubt that such stainable iron in the thyroid represents the remnants of extravasated blood from accidental hemorrhages.

A somewhat different picture is revealed in microincinerated specimens. Grafflin[18] has provided a careful account of the location of red or yellow ash in incinerated specimens of the thyroid from a Barasingha deer. The colloid is typically poor in ash and almost invariably devoid of iron, except for occasional rare follicles which exhibit a few granules. In the follicular cells, granular iron is present in the cytoplasm of many of the follicular cells. These iron granules are apically located for the most part, and are variably concentrated in different regions of the gland. Both their location within the cell and their variable distribution suggest the similar positions of the yellow fluorescent pigment of the deer thyroid. The nuclei are poor in ash and contain no recognizable iron.

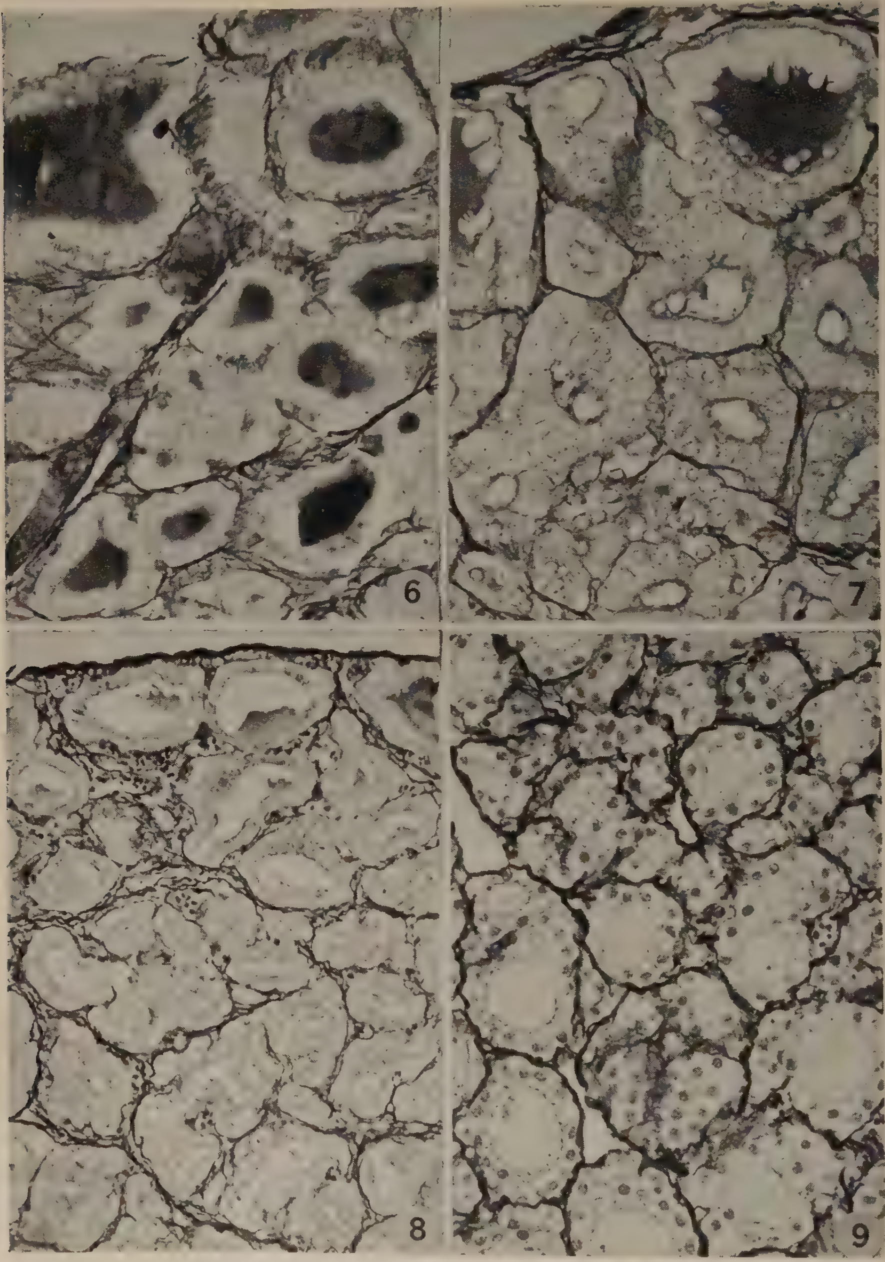
A similar situation prevails in the rat thyroid. The colloid is typically poor in ash, especially in the central, active follicles. The larger peripheral follicles, the colloid of which is denser and more basophilic, exhibit considerably greater quantities of ash, predominantly blue in color, although a few yellow or orange granules usually occur in each follicle. The follicular cells, on the other hand, contain considerable quantities of yellow ash which typically occur in the apical margins of the cells. The rat thyroid does not contain yellow fluorescent granules. Consequently, it would seem that the iron content of the follicular cells must represent some constantly occurring substance rather than reflect the presence of some adventitiously occurring pigment.

Lipoids of the Thyroid. Sudanophile material has been recognized as a regular component of the thyroid gland for many years. Langendorf[31] first described it in the follicular epithelium of the calf and dog. Fat is commonly believed to increase in amount with advancing age, although there is no complete agreement upon this point. According to Grafflin,[18] the follicular cytoplasm of the deer thyroid contains numerous small, intensely stained sudanophile droplets. The predominant position of these granules is in the apical portions of the cells. They may, however, extend down into the paranuclear region and may even be located basal to the nucleus. The picture in different follicles is surprisingly uniform, the principal variations being in the size of the lipoid droplets. From the appearance of sections stained with sudan III, one would be forced to conclude that there is only one type of cell in the follicular epithelium.

The rat thyroid gland also contains sudanophile droplets in the follicular epithelium. The droplets are predominantly located above the nucleus in a distribution similar to that described by Grafflin for the deer. Stimulation of the thyroid by subjecting rats to a cold environment or by administering thiouracil results in a prompt increase in the size and number of the sudanophile material.

Argyrophilia and Schiff Reactions of the Thyroid. In an attempt to determine the possible presence of glycogen in the thyroid, Dempsey and Singer[9] examined the glands of rats and cats which had been prepared by Bauer's[4] and Mitchell and Wislocki's[35] methods. Bauer's method involves staining glycogen, after appropriate fixation, by its reaction with Schiff's reagent. Mitchell and Wislocki showed that glycogen preserved in sections is stained an intense black by ammoniacal silver nitrate. The intrafollicular colloid stains with both procedures, although in the case of the silver reaction the colloid is colored brown rather than the jet black characteristic of glycogen.

Despite the occurrence of these reactions in the thyroid gland, the reactive substance is not glycogen. Digestion of the sections with saliva does not abolish either the Bauer or the silver reaction. Since both reactions ordinarily indicate active carbonyl groups, there is a strong likelihood that the intrafollicular colloid contains some substance, preserved by protein precipitants, characterized by reducing activity equivalent to that of aldehydes.



The sections on this plate were prepared by Pap's ammoniacal silver nitrate method. They demonstrate the nature of the reticular framework of the gland, and also illustrate the argyrophilia of the colloid. (*For description see facing page*)

The reducing activity of the colloid varies in different stages of thyroidal activity. In the glands from normal rats, the strongest reactions invariably occur in the large peripheral follicles containing the densest colloid (FIGURE 6). In the centrally located follicles, where the epithelium is higher and the colloid is less dense, the reactions are weak. Correspondingly, in animals exposed to cold (FIGURE 7) or treated with thiouracil (FIGURE 8), the reactions decline in intensity or fail completely, depending upon the degree of thyroidal activation.

In addition to the reaction of the colloid with ammoniacal silver nitrate, argyrophilic inclusions can be demonstrated in the follicular epithelium by two other silver methods. Argyrophilic granules, varying in size from dust-like in the rat to an appreciable size in the dog, are displayed in sections prepared by Bodian's protargol method (FIGURE 13). These granules are distributed rather evenly throughout the cytoplasm, being absent only in small vacuoles in the apical portions of the epithelium. With this method, the colloid is entirely unstained. Granules, similarly located in the follicular epithelium, are also revealed in sections fixed in Zenker's acetic fluid and stained by Pap's ammoniacal silver procedure. These granules exhibit no very clear alteration in number or appearance in the activated glands from rats exposed to cold temperatures (FIGURES 10 and 11).

One further comment can be made concerning the chemical behavior of the substance responsible for the carbonyl reactions of the colloid. Sections immersed directly in Schiff's reagent, which does not call for previous hydrolysis, exhibit a moderately intense violet stain in the colloid (FIGURE 12). It would appear, therefore, that the carbonyl groups are exposed and do not require preliminary unmasking by hydrolysis. Furthermore, strong acid hydrolysis, for example with tenth normal hydrochloric acid at 60°C. as in Feulgen's procedure for desoxyribonucleoproteins, lessens the intensity of the reaction or prevents it completely. The faint coloration observable in the colloid after short hydrolysis in Feulgen's procedure led Uotila[42] to suggest that desoxyribose nucleoproteins occurred within the follicle. It seems clear, from the above account of the reactions involving modifications of the Schiff reaction, that the staining of the colloid observed by Uotila did not represent a true Feulgen reaction for nucleoproteins, but was caused rather by the presence of the free carbonyl groups, which account for the Schiff reaction.

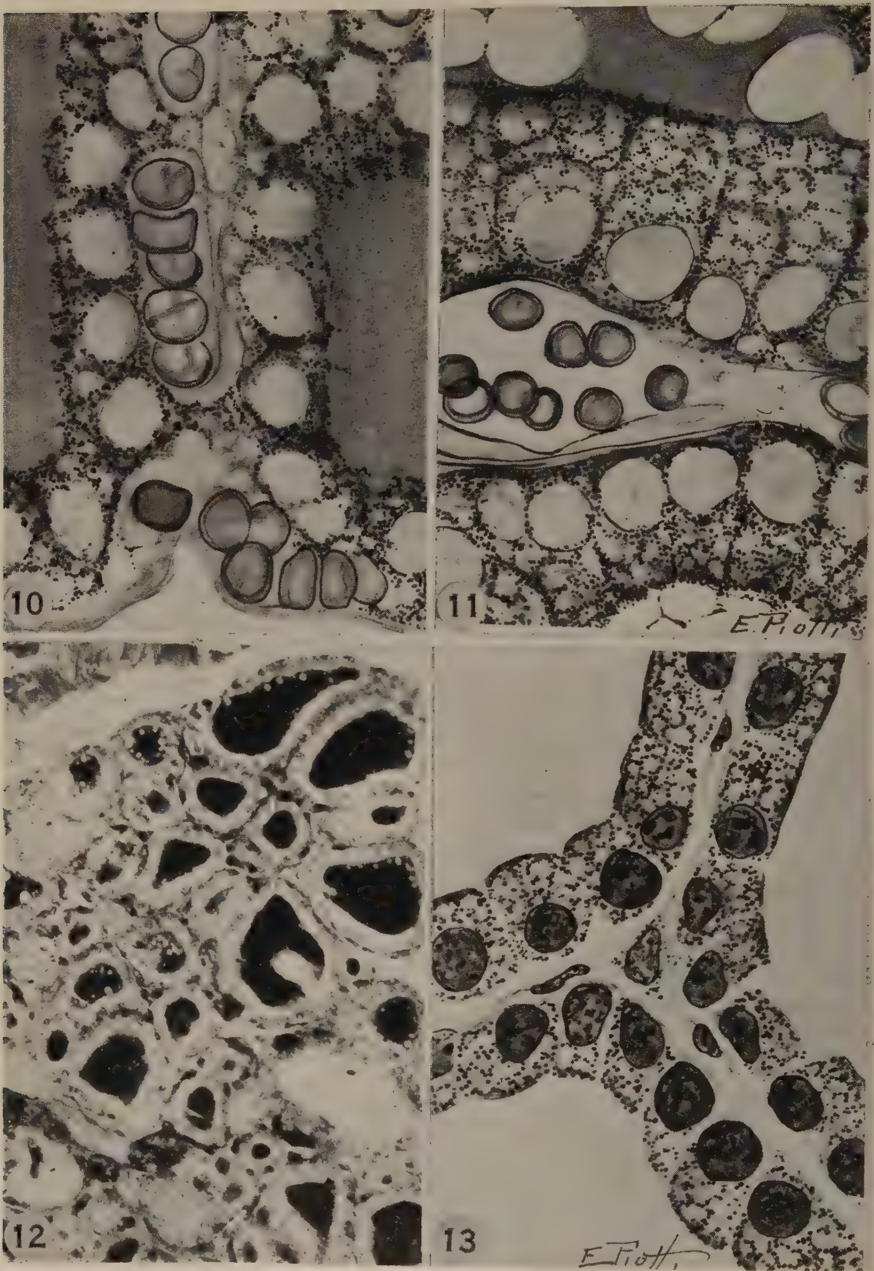
FIGURES 6–9 (*See opposite page*)

FIGURE 6. Thyroid gland from normal control rat. Bouin fixation, section digested in saliva before staining. X 350.

FIGURE 7. Section illustrating the reduction in argyrophilia of the colloid in a rat exposed to cold. Treatment of the section same as that in FIGURE 6. X 350.

FIGURE 8. Section illustrating the further reduction in argyrophilia after administration of thiouracil. Treatment of section same as that in FIGURE 6. X 200.

FIGURE 9. Abolition of argyrophilia of the colloid after fixation in formaldehyde. Cat thyroid gland. X 350.



Figures 10–13 (*For description see facing page*)

Enzyme Reactions of the Thyroid Gland. A number of enzyme systems have been located in the thyroid by chemocytological procedures. The more prominent of these involve oxidase, peroxidase, phosphatase, and proteolytic activities.

Treatment of frozen sections of rat thyroid with the nadi reagents (paraphenylenediamine and alphanapthol) leads to the rapid appearance of blue granules scattered throughout the parenchymal cells. This reaction, frequently called the indophenol oxidase reaction, has been studied by Keilin,[30] who regards it as caused by the activity of the cytochrome oxidase-cytochrome *c* system. Dempsey[7] found that the indophenol oxidase activity of rat thyroid was not abolished by the presence of the antithyroid drug, 2-thiouracil. On the other hand, using manometric methods, Franklin, Lerner, and Chaikoff[14] have reported a depression in the respiration of thyroid slices after poisoning with thiouracil and other antithyroid substances.

Peroxidase activity is also demonstrable in the rat thyroid. Blue granules appear in the follicular cells after immersion of frozen sections in mixtures of benzidine and hydrogen peroxide.[7] Unlike the nadi reaction, the peroxidase activity is easily suppressed by concentrations of thiouracil as low as one part in ten thousand. These observations have been challenged by Glock,[17] who found much of the peroxidase activity of minced thyroid tissue in a fraction containing hemoglobin. Although he was unable to identify a peroxidase characteristic for the thyroid epithelium, Glock's experiments do not disprove the possibility that such an enzyme exists. De Robertis and Grasso,[12] also using chemocytological methods, have confirmed Dempsey's observations, both as to the intracellular location of peroxidase and its inhibition by antithyroid drugs. These authors have also studied the oxidation-reduction potential of the thyroid follicle and find it greatly reduced after administration of thiourea.

De Robertis,[11] in an interesting series of experiments, has shown that a proteolytic enzyme may be demonstrated in the colloid from active thyroid follicles. The amount of this enzyme increases after the administration of thyrotropin, and causes liquefaction of the dense thyroid colloid. Such liquefaction presumably is useful in releasing the stored colloid during active secretion.

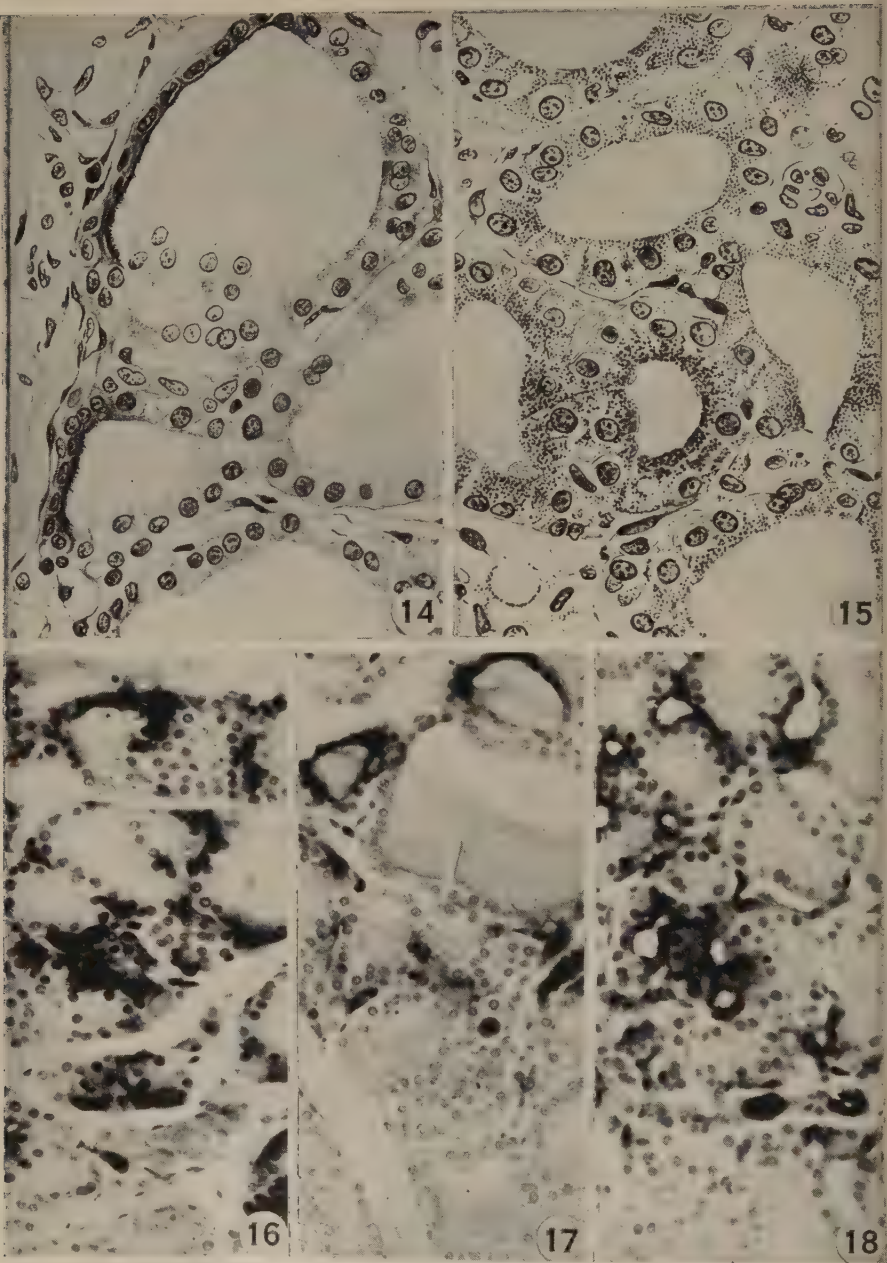
The location of phosphatases in the rat thyroid gland has been studied by Dempsey and Singer.[9] Although the concentration of neither enzyme is very high, both acid and alkaline glycerophosphatases occur in the

FIGURES 10–13 (*See opposite page*)

FIGURES 10 and 11. Argyrophilic granules in the cytoplasm of the thyroid cells from a normal rat (FIGURE 10) and a rat exposed to cold (FIGURE 11). The glands were fixed in Zenker's acetic mixture and stained by Pap's stain. The sections were drawn with a 90X objective and a 10X ocular.

FIGURE 12. Thyroid gland from a normal rat, illustrating the reaction of the colloid with the Schiff leucofuchsin reagent according to Bauer's method. X 200.

FIGURE 13. Section of the thyroid gland from a dog, illustrating the argyrophilic granules displayed in the follicular cells by Bodian's protargol method. 90X objective, 10X ocular.



The figures on this plate illustrate phosphatase reactions of the rat's thyroid gland.
(For description see facing page)

follicular epithelium. The former enzyme characteristically is located as granular deposits in the apical portions of the more active follicular cells (FIGURE 15). In the rat, such cells occur in the central follicles, whereas toward the periphery of the gland larger follicles containing dense colloid and the flattened epithelium characteristic of storage are found. These peripheral follicles are devoid of acid glycerophosphatase, but contain moderate quantities of the alkaline enzyme (FIGURE 14). After incubation in other substrates, the epithelium of the peripheral follicles exhibits alkaline phosphatases which dephosphorylate adenylic acid, yeast nuclei acid, fructose diphosphate, and glucose-1-phosphate. These substrates, unlike glycerophosphate, are not acted upon by the rat thyroid at acid pH.

In addition to the phosphatases of the parenchymal cytoplasm, reactions are also obtainable in the nuclei of the follicular cells and in the endothelium of the capillaries. The nuclei react strongly at alkaline pH when nucleic acid or glucose-1-phosphate is used as substrate. The capillary endothelium exhibits some reactivity at alkaline pH with any of the substrates mentioned, but by far the most vigorous reactions are obtained with fructose diphosphate (FIGURE 16).

The Demonstration of Iodine in the Thyroid Gland. After the discovery that iodine was an essential component of the active principle of the thyroid gland, numerous attempts were made to locate this element in tissue sections. The earlier methods devised to distinguish iodine, which have been reviewed by Gersh and Steiglitz[16] and Lison,[33] have not yielded convincing results. More recently, three methods of more critical value have been employed. Turchini[41] determined the absorption of soft x-rays by sections of the thyroid gland. Strong absorption by the colloid occurred. Since the opacity of iodine to x-rays is well known, and since little ash which might contain other absorbing elements occurs in the colloid, it seems reasonable that the absorption observed by Turchini is a function of the iodine content. This promising method should receive further investigation. The second method involves the identification of radioactivity in the thyroid after the administration of tracer doses of radioactive iodine.[25, 32] By photographic methods, the radioiodine has definitely been located in the colloid, but the definition pos-

FIGURES 14–18 (*See opposite page*)

FIGURE 14. Alkaline glycerophosphatase (pH 9.4) reaction in the thyroid of a normal rat. The section was incubated in the substrate mixture for 6 hours. The reaction is confined to the peripheral part of the outer follicles. 40X objective, 7X ocular.

FIGURE 15. Drawing illustrating the location of acid glycerophosphatase (pH 4.7) in the thyroid gland of a rat exposed to cold. The reaction did not occur in the peripheral follicles. The section was incubated in the substrate mixture for 48 hours. 40X objective, 7X ocular.

FIGURE 16. Alkaline fructose diphosphatase (pH 9.5) reaction in the thyroid from a normal animal. The section was incubated in the substrate mixture for 24 hours. Note the concentration of enzyme in the endothelium. X 200.

FIGURE 17. Thyroid gland from a rat exposed to cold, illustrating the reduction in the alkaline fructose diphosphatase reaction. X 200.

FIGURE 18. Alkaline fructose diphosphatase in the thyroid of a rat to which thiouracil had been administered. X 200.

sible with the method is so slight that the amount of iodine present in the parenchymal cells cannot yet be determined. The third method depends upon the specific absorption of diiodotyrosine and thyroxine at 2800 Å. Gersh and Caspersson[15] found that colloid strongly absorbed ultraviolet light at 2800 Å, and demonstrated further that, within the follicular cytoplasm, vacuoles occurred, the absorption in which was identical with that of the intrafollicular colloid.

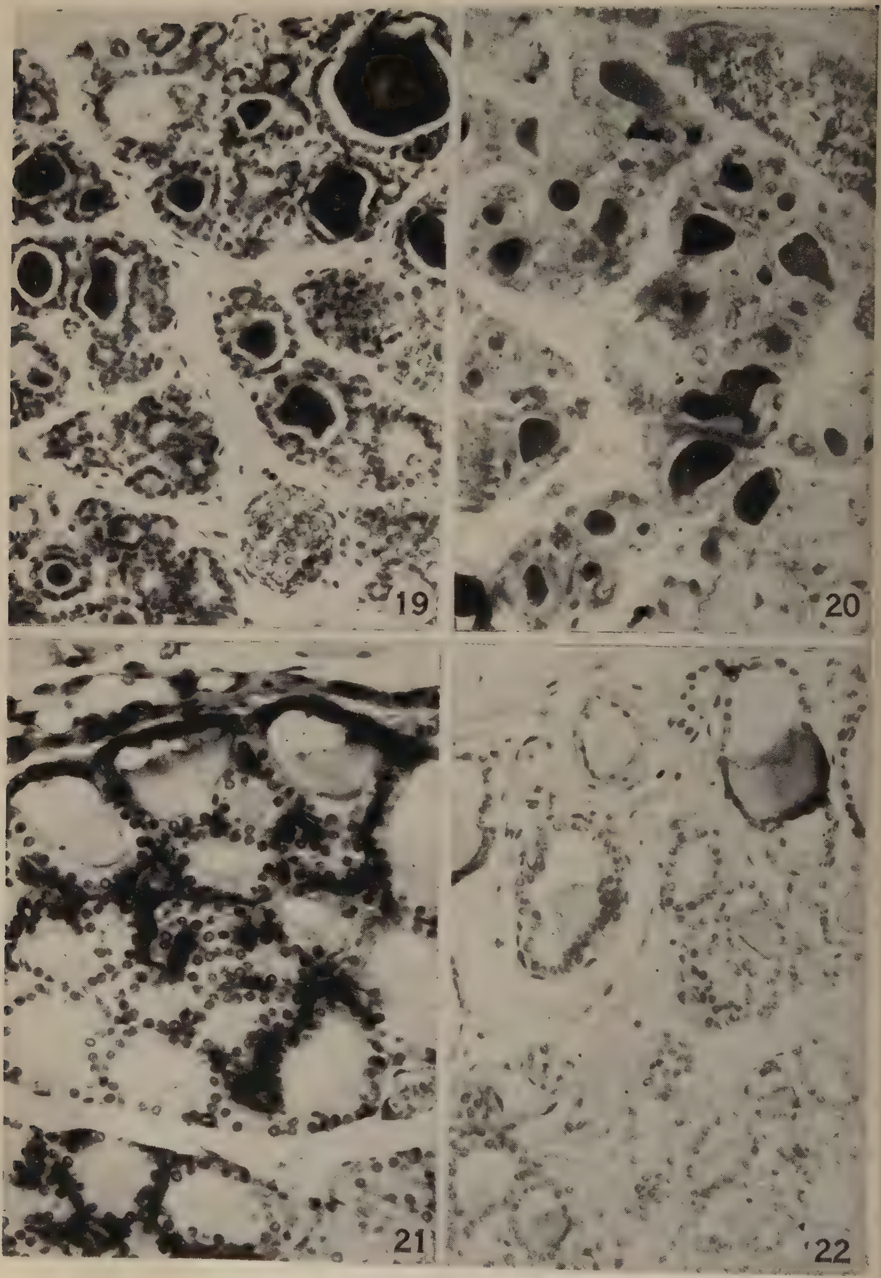
Chemocytological Changes Associated with Different Phases of Thyroid Activity. The preceding sections contain descriptions of several cytological or chemocytological reactions which differ in intensity in the peripheral and in the central follicles of the rat thyroid gland. The most reasonable interpretation of these changes is that the reactions of the central follicles are associated with active release of the thyroid hormone as compared with those of the peripheral units which are engaged in storage of the gland's active principle. Fortunately, more direct information on the state of activity comes from the study by Dempsey and Astwood[8] of the rate of secretion of the thyroid under different environmental conditions, in which it was shown that exposure to cold roughly doubled the rate of secretion, whereas maintenance in a hot environment greatly decreased the release of hormone into the systemic circulation. The cytological reactions described in the present account permit the conclusion that states of increased release of the stored hormone are accompanied by an increased height of the follicular cells, by a marked decrease in the concentration of the ribonucleoproteins responsible for the basophilia of the colloid, and by a slight decrease in the concentration of the nuclear desoxyribonucleoproteins of the follicular cells. In the colloid, concentration of reducing substances is decreased during active release of the secretion, as is also the intensity of its autofluorescence. The sudanophile droplets of the follicular epithelium increase in active states. In the capillary endothelium, the alkaline phosphatase reactions decrease in intensity (FIGURES 16 and 17), whereas in the follicular epithelium acid glycerophosphatase puts in its appearance. On the other hand, in storage or inactive states, these reactions are altered in the reverse direction.

Although the experiments involving maintenance of rats in hot or cold environments are helpful in determining the cytological correlates of increased release of the thyroid hormone, they unfortunately do not illuminate the similar correlates of synthesis or manufacture of the active principle. In other words, there is no information bearing upon the rate of hormone synthesis in the cold-stimulated animal. This difficulty can be partly overcome, however, by a different type of experiment. MacKenzie and MacKenzie[34] and Astwood, Sullivan, Bissel, and Tyslowitz[3] have shown that various antithyroid drugs prevent the formation of thyroid hormone, profoundly alter the uptake of iodine by the gland,[2] and lead to a rapid exhaustion of the stored material. The present account indicates that poisoning with thiouracil causes a series of cytological changes in the gland. The more prominent of these are an increased height of the follicular cells, a decrease in the nucleoproteins of the cytoplasm, colloid and nuclei, a decrease in the reducing substances and in the autofluorescence of the colloid, an increase in the number of sudanophile droplets in the epithelium, and an increase in the alkaline phosphatase reactions of the capillaries (FIGURE 18). Comparison of this list with the similar one in the preceding paragraph indicates that they differ in only one particular, namely, that the endothelial phosphatase is decreased during active release of the hormone but is increased after hormone synthesis is blocked. The two lists are derived from similar situations in that active release of stored hormone characterizes both, but are different in that hormone synthesis is blocked in the second and is normal in the first. It would seem, therefore, that, of all the changes listed, only the amount of endothelial phosphatase is related to the rate of synthesis of the hormone.

The cytological counterparts of synthesis of hormone can also be studied in a slightly more complicated experimental procedure. It has been shown previously that the normal store of thyroid colloid can be completely exhausted by the administration of thiouracil for a sufficiently long period. If animals in which such depletion of colloid has occurred are hypophysectomized, colloid storage again occurs,[3] whether or not thiouracil administration is continued postoperatively. The colloid formed and stored under such conditions is abnormal in that it does not contain iodine.[7] Consequently, in the glands from animals maintained in hot environments, the phenomena of storage and synthesis occur, whereas, in the thyroids from rats given thiouracil and later hypophysectomized, one observes the phenomenon of storage of colloid without synthesis of hormone. The question, therefore, arises as to the cytological differences which may be observed in these two experimental situations.

In the "inactive" glands of animals maintained in a hot room, the follicular epithelium is low, the basophilic staining of both cells and colloid is intense, the reducing activity of the colloid is high, and considerable amounts of alkaline fructose diphosphatase occur in the endothelial cells. In the glands from rats treated with thiouracil to exhaust the colloid stores and subsequently hypophysectomized, the epithelium is also low, the basophilia of the cells and colloid is intense (FIGURE 19), the reducing activity of the colloid is high (FIGURE 20), but the phosphatase reaction of the capillaries is entirely absent (FIGURE 22). It appears, therefore, that the gland in which synthesis of hormone is occurring contains phosphatase, whereas the thyroid in which synthesis is blocked does not.

The results cited in the preceding paragraphs reveal an interesting but baffling relationship between the endothelial phosphatase and the activity of the thyroid. Phosphatase cannot be directly related to hor-



Figures 19–22 (*For description see facing page*) mone synthesis, since the enzyme is elevated after thiouracil and depressed after hypophysectomy, yet synthesis does not occur in either case. Similarly, phosphatase is increased in concentration after thiouracil but depressed on exposure to cold, yet release of stored colloid characterizes both states. The enzyme is elevated on exposure to heat and depressed after hypophysectomy, although an increase in the amount of stored colloid occurs in both situations. All that can be said, therefore, is that phosphatase activity is altered in different physiological states, but in a fashion which defies correlation with the known physiological activities of the gland.

The cytological observations presented in this account indicate that an unsuspected number of products are secreted by the thyroid gland. It appears that the highly basophilic colloid which occurs during phases of storage owes its staining properties to the presence of nucleoprotein. The reducing activity of the colloid is not correlated with its hormone content, and, since reducing activity is not a characteristic of known ribonucleoproteins, presumably indicates the presence of an additional compound in the colloid. The fact that these two components continue to be formed in hypophysectomized rats to which thiouracil is administered indicates an ability of the gland to carry on complex syntheses even after poisoning with antithyroid drugs. Likewise, the active hyperplasia and hypertrophy of the gland after treatment with thiouracil indicate the ability of the thyroid cells to reduplicate rapidly their own protoplasm in a similar poisoned state. It seems likely, in view of these facts, that antithyroid drugs act at a point near the end of the metabolic chain of events leading toward hormone synthesis, rather than near the beginning where reactions supplying the materials for general cellular metabolism occur.

Summary

The cytoplasm of the follicular cells and the intrafollicular colloid exhibit basophilia after staining with methylene blue. This basophilic staining presumably indicates the presence of nucleoprotein, since it is abolished by digestion of the sections in crystalline ribonuclease and since its behavior in buffered solutions of dye is similar to that of other regions in which ribonucleoprotein is a known constituent. On the other hand, the basophilia of the colloid presumably is not caused by sulfate-containing mucopolysaccharides, since it does not stain metachromati-

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(See opposite page)

FIGURE 19. Basophilia of the colloid regenerated after hypophysectomy. Thiouracil was administered to the animal until the colloid was completely exhausted; the rat was then hypophysectomized and the colloid allowed to regenerate. Zenker-acetic fixation, eosin-methylene blue stain. X 200.

FIGURE 20. Bauer reaction of the colloid regenerated after hypophysectomy. Bouin fixation, Bauer's stain. X 200.

FIGURE 21. Alkaline fructose diphosphatase reaction in the thyroid from a normal rat. Compare with FIGURE 22. X 200.

FIGURE 22. Abolition of the alkaline fructose diphosphatase reaction in the thyroid from a hypophysectomized rat. X 200.

cally with toluidin blue and since its behavior in buffered solutions of dye is unlike that of mucus and mast-cell granules in which mucopolysaccharides are a prominent part.

Argyrophilic granules may be demonstrated in the follicular cells by Bodian's method and by Pap's stain after Zenker fixation. Argyrophilia of the colloid, coupled with positive reactions with Bauer's leucofuchsin method, indicates the presence of a substance containing active carbonyl groups.

Phosphatase reactions are exhibited by the nuclei and cytoplasm of the follicular cells and by the endothelial cells of the thyroid capillaries. Acid glycerophosphatase is present only in the central, active follicles, whereas several alkaline phosphatases occur in the peripheral follicles and in the endothelium.

The various reactions listed above are altered during different states of physiological activity. Alkaline phosphatase disappears after hypophysectomy. Basophilia and reducing activity are depressed during states of active release of the colloid, but may be formed in the hypophysectomized animal under conditions which prevent thyroid hormone synthesis.

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THE METABOLIC CIRCUIT OF THE THYROID HORMONE*

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IT is the purpose of this communication to emphasize the importance of the extra-thyroidal iodine in the body and particularly the pathways of distribution of thyroid hormone after its release from the parent gland. Intimately related to this problem is the fate of endogenous iodide released by the decomposition of the hormone which is degraded through use in the peripheral tissues. The iodide in the body is in constant circulation. As thyroid hormone is destroyed by metabolic processes in tissues, much of its inherent iodine can be recovered by the thyroid gland and reconstituted into active hormone.[1]

What is the mechanism by which the gland is able to trap iodide and concentrate it from very dilute plasma? What are the mechanisms by which the hormone is conveyed from the parent gland to various organs and individual cells? These are problems which have been neglected because general interest has been attracted to, and focused upon, the thyroid gland itself.

The Conveyance of Thyroid Hormone to Body Tissues. It is not yet settled how the hormone is delivered from the gland into the bloodstream. It seems clear that, under certain circumstances, thyroglobulin can appear both in the blood and in the lymphatics leading directly from the gland.[2, 3] It seems likely, however, that the appearance of thyroglobulin in the bloodstream is the result of trauma or inflammation in the gland and cannot be regarded as indicative of a normal physiological process. Likewise, the demonstration by immune reactions of thyroglobulin in lymphatics may be a simple reflection of experimental trauma. At the moment, therefore, we must evade this question for lack of suitable experimental evidence and take up the trail of the hormone after it reaches the blood.

The distribution of the hormone in the blood involves two questions. First, do the red cells carry any hormone? Second, with what plasma constituents is the extracellular hormone associated? With respect to both of these problems, there are still dissenting opinions, but most investigators agree that the hormone usually is precipitable along with

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For the thyroxine used we are indebted to E. R. Squibb & Sons.

For the thiouracil used we are indebted to Calco Chemical Division, American Cyanamid Company.

protein.[4, 5] Therefore, a simple coagulum of plasma protein or of the mixed proteins of laked blood will carry down all of the circulating, organically bound iodine under ordinary circumstances. Such precipitates, however, are notoriously prone to carry down smaller molecules by adsorption,[6] so that special precautions must be taken in acquiring pertinent data and scrutinizing such evidence.

With regard to the hormone in red cells, the best thing that can be said at the moment is that the erythrocytes should be avoided. Certainly, it is clear that the use of plasma alone is desirable both technically and theoretically because it is in direct equilibrium with the tissue fluids. Data from two excellent sources[7, 8] are completely in disagreement, however, on the question as to whether the red cells contain "hormonal" iodine. One group of investigators reports that the concentration in red cells is approximately that in the plasma, while another finds no evidence of a "hormonal" iodine beyond that in the plasma. It would appear that much careful work must be done on this subject, which may require more refined methods than those available at present. For example, it is difficult to separate red cells cleanly from plasma without contamination. Furthermore, the precipitation of the proteins of laked blood by heat coagulation is difficult, inasmuch as the overlapping isoelectric points of various proteins tend to interfere with complete precipitation, so that the several protein fractions must be removed *seriatim.*

There is no question, nevertheless, but that the red cells may accumulate considerable concentrations of iodide ions, especially when iodide is administered to the organism in excess. Since the pioneer work of Wallace and Brodie,[9, 10] this fact has been confirmed repeatedly. In general, the distribution of iodide between cells and plasma is similar to the distribution of the chloride ion. Normally, however, the concentration of iodide in both erythrocytes and plasma is less than 1 microgram per 100 milliliters of water. It does not, therefore, present an analytical problem of importance except when pharmacological responses are involved.

The Iodine in Plasma. As to the plasma "hormonal" iodine, considerable data have now accumulated.[11] It is well established, in man, that the concentration of the circulating "hormonal" iodine is a good index of the physiological status of the organism with respect to thyroid activity.[12, 13] Even certain cases of apparent Graves' disease can be shown not to be hyperthyroid by this technical procedure, which agrees with the best clinical appraisal performed by a well-trained physician.[14]

In recent years, it has become apparent, furthermore, that the circulating "hormonal" iodine is indeed closely related to the plasma proteins. In particular, it has been possible to isolate most of the iodine-containing material in association with one or more of the main protein fractions.[15] In the early reports by the present author, the material seemed to be associated with the smaller albumin fractions, at least in the major part.[11] When the plasma protein fractions of Professor E. J. Cohn became available, this point was pursued further and a somewhat higher concentration-peak was located in the alpha-beta-globulin fraction.[16] This latter fraction, however, although higher in specific concentration, did not account for a great mass of the total organic iodine. As the matter stands to date, the major single portion of the organically-bound iodine in the plasma resides with the albumin. The problem is under further analysis to see what the significance may be of the iodine which resides in the Fraction IV–6 of Cohn,[17] which is one of the smaller globulins.

For the moment, it might be said that these protein fractions are the sort that are expected to traverse capillary walls most readily. In the present communication, therefore, this would be the most significant finding to be emphasized with regard to these high-iodine plasma fractions. It is most likely that they are organically-bound iodine fractions, incorporated in the protein molecule. It is also conceivable, however, that one of them is free thyroxine, whereas the other fraction is incorporated into protein. These are details awaiting solution as better methods become available for handling smaller and smaller amounts of material. The analytical difficulty will be appreciated if it is realized that the most concentrated protein fractions yet obtained contain only three parts in a million. A representative series of analyses of these proteins is shown in TABLE 1. These are by no means the final results, but they indicate the nature of the problem and the experimental approach which is being pursued.

TABLE 1

IODINE IN PLASMA PROTEIN

Protein	*Fraction after Cohn*	*Protein, grams per liter*	*Iodine, µg. per liter*	*Iodine, µg./100 gm. protein*
Albumin				
small	VI	31.3	28.7	92
large	V	20.0	9.4	4.7
Globulin				
alpha	IV–2	0.7	1.2	193
beta	IV–1	1.3	1.7	139
gamma	II+III	18.5	9.0	41
Fibrinogen	I	3.6	1.95	55
Total		75.4	52.0	524.7

It will be observed, from a perusal of the fractions involved, that these might be expected to penetrate into the tissue fluid and ultimately to be collected by the lymphatics draining the tissue spaces. It is difficult, of course, to analyze pure tissue fluid, but, by comparing the content of the plasma and of the lymph simultaneously, one is able to bracket the problem and so to discover what are the probable intermediary values. It will, therefore, be interesting to pursue the problem further into body fluids in order to see what sort of relationship exists between the iodide and the protein-bound iodine of these fluids as related to the plasma from which the fluids are derived.

Protein and Iodine in Various Body Fluids. The iodide can be disposed of at once. Ordinarily, it is very low in concentration and constitutes a relatively trivial problem. It is not surprising to find concentrations of 0.5 microgram per cent in such fluids as pericardial fluid, spinal fluid, cervical lymph, and lymph from the extremities. Of course, under pharmacological circumstances, the concentration may be much higher. For example, when large doses of iodide such as used to be administered in tertiary lues are given, the plasma iodide in man may mount to concentrations of 1 or 2 *milligrams* per cent[4] and then fall off over the course of many hours toward the normal. Even an overnight fasting blood may show values above 100 micrograms per cent if a large dose of iodide has been administered on the preceding day. If the plasma protein is precipitated in such a medium, it will carry down with it a high concentration of adsorbed iodide ions and thus give a false impression of high hormonal iodine, as shown in FIGURE 1. Under such circumstances, a dialysis or some other procedure is necessary to rid the plasma protein of the tremendous excess of iodide ion before analysis of the protein.[6, 18]

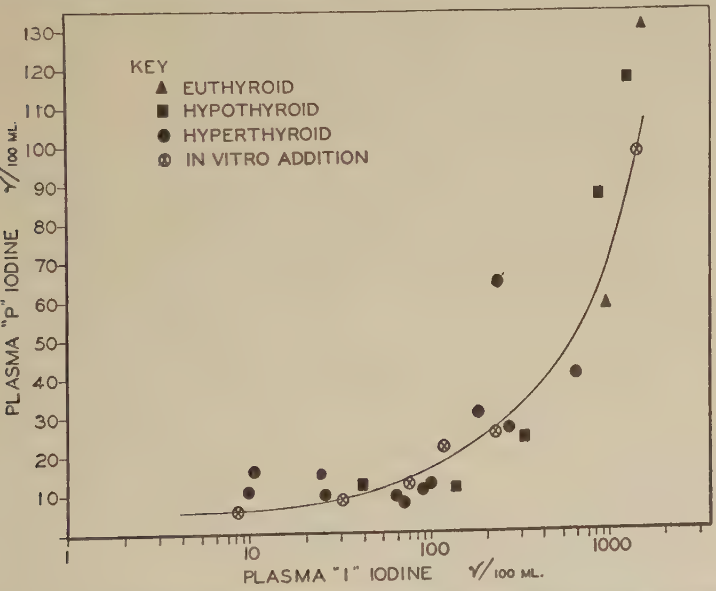

FIGURE 1. In the presence of unusually high concentrations of serum iodide (inorganic), adsorption of iodide ions upon the protein precipitate may yield spuriously high results for apparent "hormonal" iodine. (From BASSETT, COONS, & SALTER,[5] p. 522.)

The same sort of situation may obtain when iodide is introduced into the body by other means. For example, if the sodium salt of mono-iodo-methane sulfonic acid ($CH_2I.SO_3Na$), which contains 52 per cent iodine, is injected intrathecally, this material serves as a reservoir for iodine which will pay back into the plasma over long periods. With the collaboration of Dr. Donald Munro of the Boston City Hospital, this problem has been studied in our laboratory.[19] Under these circumstances, as shown in TABLE 2, values of plasma iodine may rise as high as four *milligrams* per cent within a few hours after administration. A comparison of FIGURES 2 and 3 will indicate how rapidly the exchange occurs. When the plasma so obtained is fractionated, it would appear that part of this material exists in the form of free iodide ion. However, the compound in question is supposed to undergo ionization. Therefore, on extraction with *n*-butyl alcohol, some of the iodine accumulates in the organic phase. In fact, the author obtained approximately even distribution of the material between alcohol and water. This finding suggested that at least half of the excessive iodine-containing material found in plasma under such conditions is not simple iodide but associated with an organic carrier, presumably the original "Skiodan,"[20] $CH_2I.SO_3Na$.

TABLE 2

TOTAL IODINE OF CEREBROSPINAL FLUID AND OF SERUM AFTER INTRATHECAL ADMINISTRATION OF SODIUM MONO-IODO-METHANE-SULFONATE

(Both concentrations are expressed in *milligrams* per cent.*)

Patient	*Hours after intrathecal injection* 0		½		1		2		3		18		24	
	Fluid	*Serum*	*Fluid*	*Serum*	*Fluid*	*Serum*	*Fluid*	*Serum*	*Fluid*	*Serum*	*Fluid*	*Serum*	*Fluid*	*Serum*
He	0.5				11.6									
Ya	0.0				11.1		2.8							
Whe	0.0				10.0		1.6							
Elk	0.0				9.6		5.5							
Har	0.0				6.0		3.2							
Ra	0.0				5.0		3.9							
En	0.0				4.6		2.5							
Wa		0.0		0.5				4.2				10.8		0.6
Pi			23.5		4.5	.3	4.2	0.4	2.6	0.3				
Cr				1.9	10.3	0.7	3.1	1.1						0.1
Wal		0.04		3.3		4.3		2.7						
DeF		0.02		2.7		3.4		2.7						
Chi		0.07		1.3		1.6		1.1						
La		0.01		1.1		1.9		2.9						
Pa		0.3		0.3		1.5		1.6						

* Excerpt from data of a study in collaboration with Dr. Donald Munro of Boston.
The author wishes to thank the Sterling-Winthrop Chemical Company for the sample of "Skiodan" used.

In short, for smaller iodine-containing molecules there is a constant equilibrium across body membranes, or rather a tendency to equilibrium. This tendency to equilibrium is disturbed by kinetic factors so that pure thermodynamic equilibrium probably never is completely attained. For

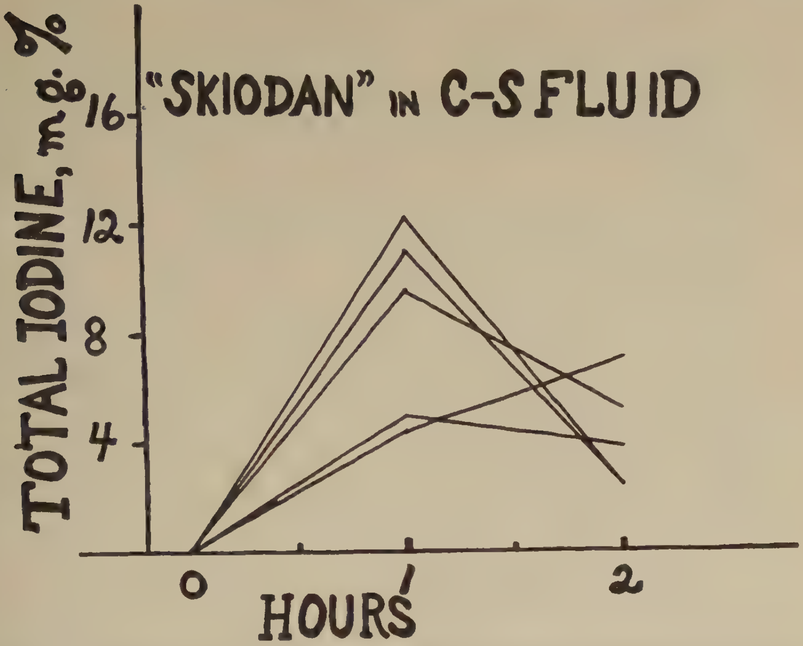

FIGURE 2. Total iodine in human cerebrospinal fluid after intrathecal instillation of sodium salt of mono-iodo-methane sulfonic acid.

practical purposes, however, one can use the thermodynamic equilibrium as a hypothetical picture toward which the fluids tend to move under actual circumstances. When the spinal fluid, to cite another example, is examined after the administration of high doses of iodide by mouth or intravenously, it is found that the concentration of iodide rises after a certain lag and, furthermore, fails to achieve as high a peak as the plasma.[21, 22]

The problem is complicated further by the fact that many organic compounds which contain iodine are constantly undergoing degradation into their constituent iodide. For example, when diiodotyrosine is administered intravenously or by mouth, it begins at once to decompose into iodide.[23] Therefore, it is questionable whether the thyroid gland itself can ever be treated by diiodotyrosine alone, except by perfusion experiments upon the isolated gland.[24]

Such dynamic considerations, however, are of minor quantitative significance, as already indicated. The important point is that throughout the body there tends to be an approximate equilibrium between the tissue fluids, provided free exchange of the iodine compounds can be achieved. Consequently, the fact that the hormone is anchored to protein carriers may be of considerable importance because, in this way, its distribution and consumption by tissue cells can be regulated. In view of the marked stimulating effect which the thyroid hormone has upon a

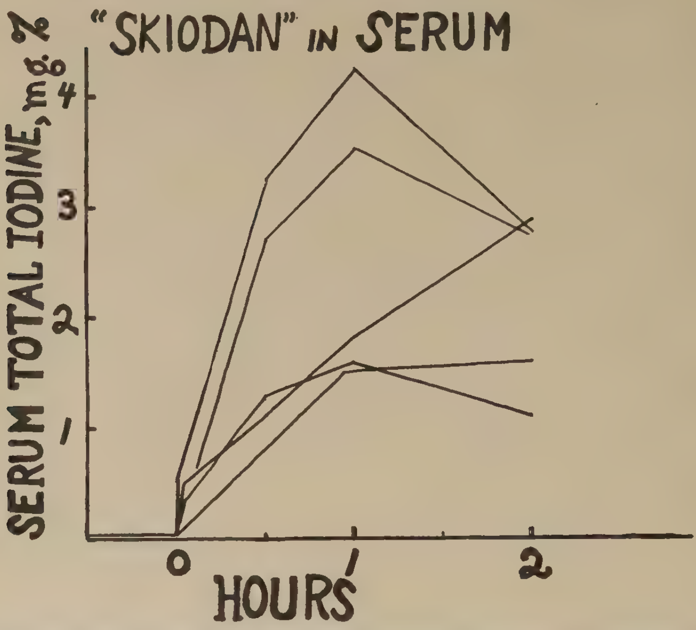

FIGURE 3. Total iodine in human blood serum after intrathecal instillation of sodium salt of mono-iodo-methane sulfonic acid. Excerpt from data obtained in collaboration with Dr. Donald Munro.

wide variety of metabolic processes, it may be a very desirable feature of its control that this very active hormone, which affects so many bodily processes, is anchored effectively to slow-moving colloidal molecules.

Through the kindness of Professor Cecil K. Drinker,[25] the author and his associates were able to obtain samples of various tissue fluids in the dog.[16] Because, in general, the dog operates at a lower concentration of "hormonal" iodine than does man, the data in question have been divided by a factor of 0.71 in order to make them comparable to human values. As shown in TABLE 3, in these animals the plasma protein and plasma iodine (including both the "hormonal" and inorganic fractions) were determined simultaneously. Out of these observations come several interesting features:

(*1*) The "hormonal" iodine of the body fluids is lower than that of the plasma.

(*2*) The protein of the body fluids is lower than that of the plasma.

(*3*) There is relatively more iodine in the clear fluids than their total protein content would indicate on a proportionate basis. In other words, the higher iodine-containing fractions of the plasma proteins apparently have leaked through the capillary wall preferentially.

(*4*) The iodine concentration of the body fluid is low and approximately that of the plasma.

If one attempts to correlate these findings, one is led to the conclusion, as a first working hypothesis, that the "hormonal" iodine is associated with those protein fractions which can more readily leak through the capillary walls into the perivascular tissue spaces. After this body of water has laved the tissue cells, it is then drained into the lymphatics, with the results shown in TABLE 3.

TABLE 3

PROTEIN-BOUND IODINE IN LYMPH*

Dog No.	*3 M*	*4 M*	*5 M*			*6 M*	*7 F*
Serum							
Protein, gm. %	8.3	8.0	7.5				
P-iodine, µg. %	4.6	9.8	4.3				6.2
Pericardial fluid							
Protein, gm. %	1.6						
P-iodine, µg. %	1.3						
Rt. cervical lymph							
Protein, gm. %	2.6	3.0				3.3	
P-iodine, µg. %	2.6	5.1				5.3	4.4
Left cervical lymph							
Protein, gm. %	2.6		2.6	2 hours later	3.1		
P-iodine, µg. %	2.8		3.9		3.8		4.0
Left forepaw							
Protein, gm. %						2.2	
P-iodine, µg. %						5.7	

* For convenience, the data for iodine concentration have been divided by the factor 0.71 in order to step up canine values to human levels.

A special case is that of the thoracic duct. This is one of the largest lymph vessels in the body. The natural tendency of the investigator is to rush to this main trunk. Unfortunately, the material which it drains comes from the gastrointestinal tract and may contain very large amounts of iodine, mostly inorganic. The results, therefore, are confusing. [Dr. Drinker has referred to this fluid privately as "bilge"!] The author agrees that one should be very cautious in interpreting the results of iodine analyses attained in this fluid, if there is any chance at all that absorption from the gastrointestinal tract may be in progress. This situation is somewhat similar to that of the bile as reported by Krayer[26] and by Zawadowsky and Perlmutter.[27] If large amounts of thyroid hormone are given intravenously or even fed to dogs over a short period of time, the thyroid iodine spills into the bile. Indeed, it may be excreted so rapidly that it may even escape destruction by the liver and actually may be recognizable as potent hormone in the biliary excretion. Obviously, such findings constitute a special case which might be called "detoxification" and should not be classed with concentrations which represent a working equilibrium between various organs.

There is still much to be learned concerning the means by which the thyroid hormone is associated with the plasma fractions. Thyroxine, being an amino acid, conceivably may be built into the body of the circulating protein just as any other amino acid is so constituted. Indeed, the work of Schoenheimer[28] and his school has indicated that there is a constant flux of metabolites in the organism, so that any single measurement reflects merely the status of the so-called "metabolic pool" at that particular instant. There is also the possibility, raised by Cohn,[29] that hormones and enzymes are carried in the plasma fractions by some sort of co-valent bonds. These co-valent bonds would be sufficiently strong to prevent removal by ordinary dialysis and perhaps to prevent separation in certain organic solvents. Such an association, however, would not necessarily imply a peptide linkage. These are problems which will be settled more readily when the work which is going on in collaboration with Dr. Cohn's laboratory has been extended further.

The Iodine in Tissues. There are two general problems concerning the regulation of the iodine in tissues. The first concerns the entrance of iodide; the second, the admission of "hormonal" iodine. Furthermore, the problem must be subdivided with respect to specific tissues involved. Obviously, those tissues which have a specific mechanism for trapping iodide—presumably by the formation of a new chemical substance—will react somewhat differently than those tissues which have no such specific iodine-bearer.

With regard to the inorganic fraction, the distribution of iodide in body tissues at large seems to have been well summarized by Wallace and Brodie,[9] who showed that the general distribution was rather similar to that of the chloride ion. They demonstrated, for example, that the ratio of such ions in plasma water and in tissue water was approximately the same for both chlorides and iodides. The principal exception was brain tissue, in which chloride seemed deficient relative to plasma.[10] The great exception to this general picture, obviously, is the thyroid gland itself. It still remains a considerable puzzle how the thyroid is able to trap iodine from such low concentrations as exist in the plasma. Nevertheless, certain suggestive results have been obtained in the author's laboratory in rats treated with thiouracil over the course of weeks and months. In a previous communication,[30] it was shown that, when iodide was given together with thiouracil in high concentration, the glands of such animals contained as much iodine as a normal gland. Of course, the concentration was lower than normal because the gland had increased in bulk through hypertrophy and hyperplasia. When such glands were fractionated, it appeared at first sight that most of the iodine was apparently "inorganic" in type. This result has been reported from a number of laboratories.[31, 32]

The author, however, raised the question whether this "iodide," so-called, may not indicate an intracellular enzymic mechanism at work within the hypertrophied tissues. Accordingly, after 8 months of treatment with these thyroid blocking agents, the animals' glands were removed, extracted, and analyzed after certain chemical preliminary pro-

Specimen Protocol for Table 4

Group B rats received a standard dog chow such that 16 grams contained 13 micrograms of iodine. This was the approximate daily ration which the animals consumed during the earlier part of the experiment.

Group G rats were fed the same chow reinforced with thiouracil and potassium iodide. The formula for the diet was

ground dog chow	983.6 gm.
thiouracil	10.0 gm.
KI	6.4 gm.
	1,000.0 gm.

Group E rats were fed chow reinforced with potassium thiocyanate and potassium iodide. The formula for the diet was

ground dog chow	973.6 gm.
KSCN	20.0 gm.
KI	6.4 gm.
	1,000.0 gm.

The protocol for rat no. 25G follows:

This male rat was started on the diet containing thiouracil and potassium iodide on April 26, 1945. At that time, he weighed 250 grams. He was autopsied on March 27, 1946, at which time he weighed 383 grams. The body length from nose to breech was almost exactly 9 inches. The tail was 8 inches long. The pituitary weighed 15.7 milligrams; the thyroid 298.8 milligrams. One lobe of the thyroid was preserved in the formalin fixative at the same time that the pituitary was preserved in a Zenker fixative.

The other lobe of the thyroid, weighing 157.2 milligrams, was homogenized in dilute ammonia and the resulting suspension diluted to 4.0 cc. Each of the four 1-ml. aliquots was used for iodine determinations as indicated in the table. The total iodine was determined, the iodine after dialysis for twenty-four hours, and the heat-coagulable iodine. The last aliquot was brought to pH 6.0 and treated with 15 volumes of acetone. After standing for twenty-four hours, the resulting protein precipitate was separated, and the iodine determined both in the precipitate and in the supernatant fluid. The results are indicated in TABLE 4.

Simultaneous determinations were made of the iodine in skeletal muscle from the thigh region. In some samples, the total iodine content was determined directly. Other samples were homogenized, subjected to heat coagulation, and the respective iodine contents in the coagulum and the supernatant fluid determined separately.

cedures. Some of the pertinent data are given in TABLE 4. It will be observed that, if the extracted gland substance is subjected to heat coagulation, much of the iodine can be removed by this procedure. These conclusions, at first sight, might lead to the assumption that the iodine consisted simply of dissolved iodide. Certain other considerations, however, open up the possibility that something more complex is occurring. The central feature of these experiments is the indication that the "iodide" is bound to some albuminous constituent of the cell or gland. For example, if iodide is added to a homogenate containing the same amount of extract from the normal glands, it is practically all lost in the course of 24 hours under certain conditions of dialysis. If, on the contrary, the same amounts of thyroid homogenate and of iodine from such a hypertrophied gland are selected in one sample and treated similarly, something like half of their iodine remains within the dialyzing membrane after the same period of time.

This finding suggests that there is present, in these hypertrophied

Table 4

Iodine Fractions in Fresh Thyroids of Male Rats Treated with Goitrogens

Rat No.	*Wt. of rat, gm.*	*Wt. of thyroid, mg.*	*Fraction of homogenate*	*Iodine in thyroid (micrograms)*			
				Total iodine	*After dialysis*	*Heat coagulable*	*P'pt'ed by acetone*
14 B							
Control	270	32.0					
	to		Protein		0.3	0.7	0
	327*		Iodide			2.5	3.0
			Sum	*2.7*		*3.2*	*3.0*
21 G							
Thiouracil							
plus KI	220	167.1					
	to		Protein		2.0	2.7	4.7
	296		Iodide			8.3	6.9
			Sum	*11.5*		*11.0*	*11.6*
25 G							
Thiouracil							
plus KI	250	298.8					
	to		Protein		3.2	3.0	6.1
	383		Iodide			9.6	7.9
			Sum	*14.0*		*12.6*	*14.0*
22 E							
Thiocyanate							
plus KI	290	35.9					
	to		Protein		1.5	3.5	0
	376		Iodide			2.4	4.1
			Sum	*3.3*		*5.9*	*4.1*

* Weights are given for the beginning and the end of the eight-month period during which the animals were under study.

glands, an expanded biochemical system which has a high affinity for iodine. A similar result is achieved when, instead of dialyzing and heat coagulating, one subjects the extract of the hypertrophied thyroid to precipitation by acetone at pH 6. Under these circumstances, it is possible to precipitate iodine-containing protein material, as shown in Table 4. When normal thyroid tissue is treated similarly, on the other hand, the amount of iodine-containing protein so precipitated is almost negligible. At present, these experiments can be regarded only as suggestive because there may be differences in the relative water and protein contents of the normal and the hypertrophied glands, respectively, which might affect their physico-chemical behavior. Perhaps, also, the thiouracil present may serve to mordant the iodine to the colloidal carrier.

Therefore, the suggestion should merely be raised that, when hyperactivity is induced in a thyroid by thiouracil, there occurs concomitantly with visible cellular proliferation a hypertrophy of the constituent enzyme systems. These so-called iodases are concerned with the trapping of iodine and its subsequent synthesis into diiodotyrosine. This increase in the mass of intracellular enzyme makes available a larger amount of colloidal carrier upon which iodide ions can be trapped. In this way, when iodide arrives inside the follicular cells and conceivably also within the thyroid follicle, a part of the dissolved iodine will become associated with colloid carriers and part of it will remain free. Indeed, in connection with the titration curves of proteins and the calculated acid-binding capacity thereof, it is a routine procedure for physical chemists to correct the amount of acid added for the free or ionized portion of the anion.[33] In other words, in the titration of a protein with hydriodic acid, due cognizance would routinely be taken of "free" and "bound" iodide, conveniently designated as fractions I_F and I_B, respectively. Obviously, such an enzyme complex, in order to be effective physiologically, would participate in a change in the state of oxidation of the iodide ion to a state which is equivalent to elementary iodine. It is conceivable, therefore, that such iodine-conveying systems may contain both an iodinase and a peroxidase component. This possibility arises by analogy with the well-known enzyme systems which convert molecular oxygen into oxygen available for biological oxidative processes.[34] Much more work obviously must be done along these lines before one can speak definitely. Keston[35] has offered an important model of this sort by using the xanthine oxidase of milk to form thyroxine. It is evident from TABLE 4 that thiocyanate prevents the initial trapping of iodide by the colloid, iodinase. Thiouracil, however, acts at a later stage in the iodine economy of the thyroid gland.

The Trapping of Radio-Iodide by the Thyroid. Radioactive iodine has been used for three main purposes in connection with the thyroid. In the early experiments, such as those of Hamilton and Soley[36] or Hertz, Roberts, and Evans,[37] labeled iodide was used. The results so obtained obviously indicated the effect and fate of the iodide carrier used, and were readily evaluated. With the advent of highly radioactive isotopes, however, the "tracer" dose has been used extensively. Unfortunately, it is uncommon to find in the literature simultaneous analyses of total iodine by classical microchemical methods. This second type of application of radio-iodide, therefore, has led to much confusion, because many investigators have failed to realize how important it is to determine *specific* rather than *absolute* radioactivity in tissues. Consequently, many of the "tracer dose" experiments cannot be interpreted in physiological terms. The problem is further complicated by the possibility of atomic interchange ("the exchange reaction"), as discussed by Salter.[12]

The third use of radio-iodide has been for destructive purposes, as described by Hertz and Roberts[38] and Chapman and Evans.[39] This application has led to a surprising effect on iodine balance, especially when applied to certain cases of thyroid neoplastic disease in which the total mass of metastatic tissue was large. In collaboration with Dr. F. Homburger[40] of the Memorial Hospital, the author has had the opportunity of studying the effect of such therapy upon the circulating iodine. Only two short excerpts from the data can be cited here. One patient, whose estimated metastatic tissue approximated two kilos, went into "thyroid storm" after the administration of highly radioactive iodide. Although the total energy so delivered was large, the amount of iodide administered was less than a microgram. Nevertheless, the patient's circulating "hormonal" iodine rose to 26 micrograms per cent and the inorganic fraction to 560 micrograms per cent. Obviously, by the dissolution of the several masses of thyroid tissue, excessive amounts of thyroxine and of iodide had been released into the circulation. Another patient showed temporary hyperthyroidism, followed by hypothyroidism, as destruction or inhibition of thyroid tissue approached completion. This reversal of thyroid status is illustrated by the short series of analyses in TABLE 5.

TABLE 5

SERUM IODINE VALUES AFTER TREATMENT WITH HIGHLY RADIOACTIVE IODIDE*

	Date	*"Hormonal" iodine μg. per cent*	*Iodide μg. per cent*
Before treatment	12–3–46	4.7	1.6
After treatment	12–10–46	12.1	8.6
	12–17–46	10.4	2.4
	12–24–46	4.1	2.7
	12–31–46	3.8	2.0
	1–2–47	4.1	3.4
	1–7–47	6.3	7.0
	1–14–47	5.0	12.8
	1–21–47	4.1	1.8
	1–28–47	4.5	14.2

* The arbitrary normal limits of "hormonal" iodine are considered to be 4.0 and 8.0 micrograms per cent.

"Hormonal" Iodine in Tissues. In considering the organically bound iodine in tissues, one must consider two specific cases. The first is the high concentration of iodine reported in certain endocrine glands, notably the pituitary and the ovary. This problem will be passed by in the present communication, except to note that Courrier[41] has reported that radio-diiodotyrosine is fixed specifically by the anterior pituitary of rabbits, whereas a similar amount of radioactive potassium iodide produces no such accumulation. Therefore, Courrier would agree with the older investigators who stated that the pituitary had the capacity for concentrating iodine and particularly for trapping thyroid hormone. The ovarian iodine, as reported by Perkin,[42] is also high in the presence of an actively functioning thyroid and tends to fall in myxedematous animals. It still remains to be shown whether the iodine in these glands is derived from prefabricated thyroid hormone or whether these glands themselves have some property of synthesizing an organic iodine complex. Moreover, the high lipoid content of these organs tends to yield high false values for iodine. Therefore, further study by modern analytical techniques are needed before physiological conclusions can be drawn.

With regard to the second case, namely, that of iodine in the tissues at large, the problem seems to center primarily in skeletal muscle. It has long been known that the bulk of the body iodine is located in skeletal muscle and, exclusive of the thyroid, over three-quarters of it is so confined.[43] Many years ago, Kommerell[44] studied the effect of feeding flesh from normal dogs as against flesh from thyroidectomized dogs. He reported that the latter sort of meat produced no "specific dynamic action" of protein as expected. It still remains debatable what this finding signified, but other investigators since then have shown that after thyroidectomy the stores of iodine within the body decline.[45] Moreover, at the same time, the urinary excretion of iodine increases because no trapping mechanism is available for the reconversion of the iodide into hormone. If one is willing to assume that the extra iodine excreted in such metabolic studies represents degraded hormone, one can calculate the amount of hormone originally held in body muscle. It is now becoming possible to confirm such computations by the direct analysis of skeletal muscle.[46] In brief, it seems clear that, in frank myxedema, uncomplicated by large doses of exogenous iodine, there is very little iodine within the muscle. Indeed, the total iodine of the muscle approaches that to be predicted from the circulating iodide and is probably less than 1 microgram per cent. In fact, some analyses show bare traces of iodine, even when one uses analytical techniques adapted to the analysis of "trace" elements.

On the other hand, in animals made hyperthyroid by the administration of hormone of *exogenous* origin, the iodine in the peripheral tissues increases perceptibly.[47] In general, one may say that there is a direct relation between the concentration of circulating plasma hormonal iodine and that of the tissues. The real problem, therefore, is the quantitative one, namely—How does the muscle-fixed iodine vary with respect to the plasma-fixed iodine? The answer to this quantitative problem is plagued by several technical difficulties. First of all is the difficulty of analyzing very small amounts of material. (In the author's laboratory, it has been possible, however, to obtain satisfactory results in rats with amounts of muscle approximating 250 milligrams.) The second problem is that of separating inorganic from organic iodine. If the iodine of the environment and, in particular, of the ingesta is low, this problem is minimized. When iodide is administered, however, a major problem of analysis is involved, as shown in TABLE 6. It is especially difficult to rid tissue extracts of adsorbed iodide ions, and fantastic values for "hormone" iodine may be attained. The next difficulty is that of species. Indeed, McClendon[48] has suggested that in the smaller mammals which (as it were) live faster, the bound iodine of the peripheral tissues runs higher. He gives such values, for example, as 6 micrograms per cent in the steer as against 24 micrograms per cent in the mouse. There are, as yet, not enough data available to test this statement. It is true, however, that the dog and the rat have lower values for circulating plasma "hormonal"

TABLE 6

TOTAL IODINE CONCENTRATION IN FRESH RAT TISSUES
(MICROGRAMS PER CENT × 2)*

Rat No. normal	*Brain (hypothalamus)*	*Heart*	*Kidney*	*Liver*	*Muscle*	*Serum*
1	11	15	8	9	8	3
2	6	10	9	18	6	
3	12	12	8	19	9	
4	13	11	9	12	8	
5	15	12	8	12	8	
6	10	11	10	14	9	
7		8	10	13	10	
8	13	12	6	10	8	
9	16	22	10	12	10	
10	13	26	17	(33)	16	
11		13	8	17	13	
12	11	23		18	11	
13	10	7	6	10	6	
14	8	15	13	10	9	
15	26	24	15	(33)	10	
Average	*12.6*±1.3	*14.0*±1.6	*9.7*±0.9	*13.4*±1.0	*10.7*±0.7	
Treated with tracer iodide						
16	8	11	9	16	11	

* The variation in these data is probably due more to analytical technique than to variation in animals. Note that these relative values are twice the actual findings.

iodine than does man. One must be prepared, therefore, to find variations in the tissue iodine.

There is another problem to be solved with respect to the localization of iodine within tissues: Does the thyroid hormone operate only at the cell membrane, or does it penetrate the cell to become an integral part of a tissue enzyme mechanism? Should one be content to express "hormonal" iodine concentrations in tissues in terms of the fresh tissue, or should one extract the fat and reduce the iodine content to dry weight or even to phosphorous content? What is the effect of varying water content in the tissues? These many factors will have to be controlled, one by one, before a definitive answer to the question of tissue iodine can be reached.

At the present time, the best one can do is to state that there are not enough data to confute the previously published conclusion that the concentration of "hormonal" iodine in tissues is a logarithmic function of the basal metabolic rate.[11] Such a conclusion, at present, is based solely upon maintenance levels of metabolism. By integrating the decay curve with respect to time, one arrives at the conclusion before stated.[1] It is difficult to believe that such a conclusion can be fundamentally wrong. Rather, it seems that the chief contribution of careful tissue analysis will be a more accurate evaluation of the constants involved in equations such as those suggested by Boothby and Baldes[49] many years ago.

There is still another problem, however, which is of fundamental import. This is the actual state of the organically-bound iodine within peripheral tissues. At the moment, the author is struggling with the isolation of plasma fractions and only a few preliminary experiments have been undertaken with skeletal muscles, as shown in TABLE 6. It appears, however, that muscle iodine, like plasma iodine, can be divided into two fractions, the first inorganic, the second protein-bound. Furthermore, it is possible to separate the muscle sap into fractions which are respectively low and high in relative iodine content. Eventually, as these experiments are pursued further, it should be possible to gain a better conception of the function of the thyroid hormone in the peripheral cell. Already it has been shown that muscle, kidney, and liver contain colloidally-bound iodine. In extracts of muscle from man, cat, and rat the protein-bound iodine can be separated into several protein fractions of different iodine contents.[50] In myxedematous rats this colloidal iodine vanishes; in hyperthyroid rats it rises to values several times the normal range.[51] The colloidally-bound iodine does not behave like free thyroxine added to muscle extracts. Therefore, this colloidal material has been named "thyrenzyme," because it appears analogous to co-carboxylase in relation to thiamine.

Summary

Throughout the mammalian body, there is a continuous circulation of iodine in two main forms, namely, as iodide and as thyroid hormone. Whether other forms of organically-bound iodine exist normally is unknown, but when such compounds are encountered under pharmacological circumstances they also circulate in a characteristic fashion. The rather free movement of these organic and inorganic types is controlled by specific mechanisms which fix them in characteristic sites. The inorganic iodine is trapped and concentrated in the thyroid gland after abstraction from plasma which usually is very poor in iodide. There is no definite evidence that any other tissue in the body can effect so marked a concentration. The thyroid hormone, in the second instance, is fixed in two general locations. The first of these is the doubtful preferential accumulation in the anterior pituitary and the ovary. The second is the fixation of the hormone in peripheral tissues, notably skeletal muscle.

The conveyance of the two types of iodine differs. Iodide is evenly distributed throughout all body fluids and tissues including the brain. The "hormonal" iodine, however, is associated with certain fractions of the plasma proteins. As these colloidal fractions filter through capillary walls, they reach the tissue spaces and lave the cells before being drained into the lymphatics. The nature of the protein complex in muscle and other peripheral tissues which bind "hormonal" iodine is still largely unknown. This combination, however, seems to be concerned with the activity of tissue enzymes. When the tissue hormone is decomposed through use, it yields up iodide—and the cycle starts afresh.

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Discussion of the Paper

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Dr. Salter has referred to results which have been observed after administration of Skiodan intraspinally. It should be pointed out that Skiodan is not ordinarily injected in this way, nor is the solution designed for this form of administration. It is hypertonic and may cause irritation which can best be minimized by the previous induction of spinal anesthesia.

In view of the obvious difficulties of using an agent which requires previous anesthetization of the structures to which it is to be applied, it would seem to be desirable to emphasize that Skiodan should be injected intraspinally only under special circumstances and where there are persuasive reasons why this procedure must be used. It is not a diagnostic procedure to be used routinely, and in all probability it should be restricted to those who are highly skilled in its administration, and to cases where no safer alternative is available.

STUDIES ON
THE FORMATION OF ORGANICALLY-BOUND IODINE COMPOUNDS IN THE THYROID GLAND AND THEIR APPEARANCE IN PLASMA AS SHOWN BY THE USE OF RADIOACTIVE IODINE

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The Formation of Diiodotyrosine and Thyroxine by the Normal Thyroid Gland

THE work of many investigators has made it clear that 90 per cent or more of the iodine in the normal thyroid gland is present in organic combination. This organic iodine, according to Harington,[1] can be completely accounted for by thyroxine and diiodotyrosine. These two compounds do not normally exist free in the gland but can be isolated after suitable hydrolysis of the thyroid protein.

The application of radioactive iodine as a labeling agent provided for the first time a convenient method for measuring rates of formation of diiodotyrosine and thyroxine by thyroid tissue.[2] By this means, molecules of diiodotyrosine and thyroxine can be "dated," thus permitting a distinction to be made between molecules that were newly formed and those that were present before the introduction of the isotope into the body.

The radioisotope of iodine, I^{131}, which has a half-life of 8 days, has proved most useful for biological studies. It has been prepared by deuteron bombardment of tellurium in the cyclotron[3] and more recently has been obtained as a fission product of U^{235}.[4] Other radioactive isotopes of iodine (I^{128}, I^{130}), because of their short half-lives, are less useful for metabolic studies.

The present section deals with experiments in which the formation of diiodotyrosine and thyroxine by the thyroid gland of the intact animal was studied with the aid of I^{131}, which will hereafter be referred to as I^*.

The Rate at which Administered Inorganic Iodide is Organically Bound by the Intact Thyroid Gland. In the experiment recorded in TABLE 1, rats were injected intravenously with a tracer* dose of radio-iodide. The thyroids were removed at intervals of 15 minutes to 50 hours after the injection and homogenized with cold 10 per cent trichloroacetic acid. The homogenate was centrifuged and washed once with trichloroacetic acid. The residue was hydrolyzed and then separated into thyroxine and diiodotyrosine fractions by a method previously described.[5]

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* The term "tracer dose of radioactive iodine" as used here refers to a sample of I^{131} containing negligible amounts of the naturally-occurring stable iodine isotope I^{127}.

As early as 15 minutes after the injection, 95 per cent of the radioactivity present in the thyroid was precipitable with trichloroacetic acid and therefore presumed to be organically bound. At this early interval, the greatest part of the activity, about 80 per cent, was already in the diiodotyrosine fraction, whereas 10–15 per cent was in the thyroxine fraction.

How the Gland's Iodine is Distributed between Thyroxine and Diiodotyrosine. It is well known that diiodotyrosine constitutes the largest fraction of the gland's total iodine. The exact values ascribed to the diiodotyrosine content depend upon the procedure used for the separation of thyroxine from diiodotyrosine. The acid-separation procedure of Harington and Randall[6] yields lower values for the diiodotyrosine content of the gland than does the butyl-alcohol fractionation procedure of Leland and Foster[7] or of Blau.[8]

The data in TABLE 1 were obtained with the aid of a butyl-alcohol separation procedure adapted in this laboratory to small quantities of thyroid tissue such as that present in a single rat.[5] The distribution of the gland's I^{127} between thyroxine and diiodotyrosine was found to be strikingly constant. The percentage of the gland's iodine present in the thyroxine fraction varied from 23 to 29; the mean was 26, and its standard error 0.4. This finding agrees well with previous reports from our laboratory in which it was demonstrated that not only is there a constant percentage of iodine present in the gland as thyroxine, but this percentage remains constant even when the total gland iodine is augmented by increasing the iodine intake.[9]

In these same rats, there was also a fairly constant distribution of *radio-iodine* between the thyroxine and diiodotyrosine fractions of the thyroid at each interval. This is not surprising in view of the constancy in the distribution of naturally-occurring I^{127} between these two compounds.

Specific Activity-Time Relations as Evidence of the Metabolic Interrelations between Thyroxine and Diiodotyrosine. Harington[10] has recently reviewed the evidence in support of the view that diiodotyrosine is the biological precursor of thyroxine in the thyroid gland. An important link in this chain of evidence is provided by studies in which the specific activities* of thyroxine iodine and diiodotyrosine iodine were compared at different intervals after the injection of radio-iodine. Such an experiment was first carried out by Mann, Leblond, and Warren,[11] who injected 6 dogs intravenously with a tracer dose of radioactive iodine and sacrificed them in groups of 2 at the following intervals: 0.5, 8, and 48 hours. The uptake of radio-iodine by the thyroids of these dogs varied

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* Specific activity in this case denotes the number of radioactive units of iodine found in a given fraction per unit weight of non-radioactive (*i.e.*, total) iodine in that fraction.

TABLE 1

THE I* AND I127 CONTENT OF THYROXINE AND DIIODOTYROSINE FRACTIONS OF THE RAT THYROID AT VARIOUS INTERVALS AFTER THE INTRAVENOUS INJECTION OF A TRACER DOSE OF RADIO-IODIDE

*Interval after injection of I**	*Per cent of administered I* recovered in thyroids*			*Per cent of Total thyroid I* found in*		*Thyroid I^{127}*			
						Thyroxine fraction		*Diiodotyrosine fraction*	
	Total iodine	*Thyroxine fraction*	*Diiodo-tyrosine fraction*	*Thyroxine fraction*	*Diiodo-tyrosine fraction*	*Amount* *µg.*	*Per cent of gland's total I^{127}*	*Amount* *µg.*	*Per cent of gland's total I^{127}*
15 min.†	1.6	0.20	1.3	12.5	82.7	3.4	27	8.7	69
1 hr.†	3.9	0.68	3.1	17.5	79.8	3.1	26	8.5	70
4 hrs.†	8.4	1.6	6.6	19.1	79.0	2.9	26	7.6	70
14 hrs.†	19.1	4.9	13.9	25.1	73.1	4.3	28	10.4	67
50 hrs.†	12.2	3.3	8.7	27.0	71.4	2.7	25	7.8	71

† Each line of figures is the average of closely agreeing analyses carried out separately on 3 or 4 rats.

so greatly that it was difficult to compare directly the specific activities of the various fractions in the different dogs. In order to overcome these variations, the specific activity of each iodine fraction of a given dog was calculated as a percentage of the specific activity of the total iodine. The latter values, designated "relative specific activities," were then plotted against time.

There are several aspects of the experiments of Mann *et al.* that are open to criticism. These have been discussed in some detail elsewhere.[12] It need only be pointed out here that most of their difficulties arose from the use of a variable experimental animal, the dog.

The use of a more uniform group of animals such as rats (TABLE 1) obviated most of these difficulties. Since the specific activities of a given iodine fraction agreed fairly well for the rats in each group, it became feasible to plot the averages against time, as shown in FIGURE 1. This enabled us to apply the method devised by Zilversmit *et al.*[13] for determining whether one compound in a tissue is a precursor of another. FIGURE 1 shows that the specific activity-time curves of thyroxine iodine and diiodotyrosine iodine follow the general pattern of a precursor and its product as derived by these workers. Thus, the specific activity of the diiodotyrosine iodine remained higher than that of the thyroxine iodine until the maximum of the thyroxine specific activity was reached, at which time the specific activity of the diiodotyrosine iodine fell below

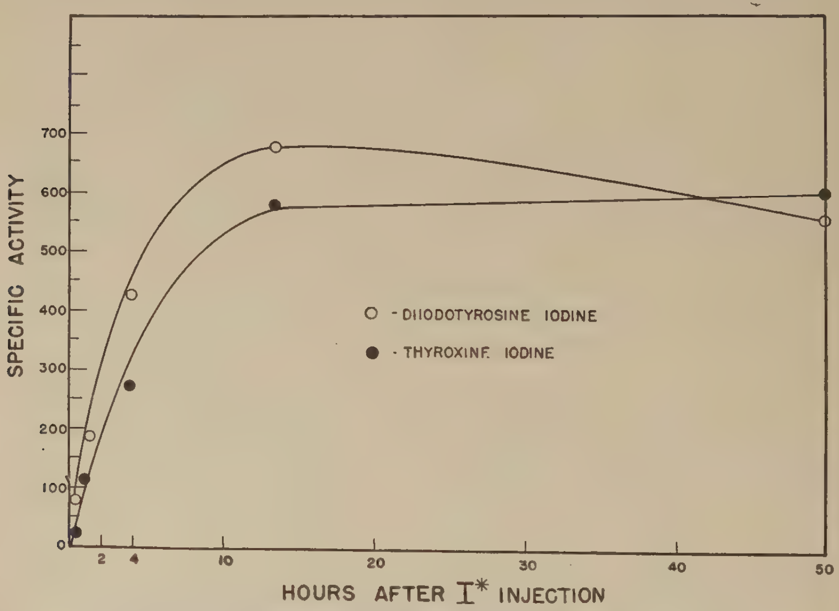

FIGURE 1. Specific activity-time relations of thyroxine iodine and diiodotyrosine iodine in the thyroid glands of rats injected with a tracer dose of I*.

that of the thyroxine iodine. This may be taken as strong evidence that diiodotyrosine is the natural precursor of thyroxine.

It was shown by Zilversmit *et al.*[13] that curves of the type shown in FIGURE 1 can also be used to calculate the time required for the complete renewal of thyroxine in the gland (turnover time). When this calculation was carried out with the data recorded in FIGURE 1, the turnover time of thyroxine amounted to approximately 24 hours. This means that the thyroid gland secreted every 24 hours an amount of thyroxine equal that present in the gland. Since the average thyroxine iodine content of the thyroid gland was 3.3 micrograms, the rate of thyroxine iodine secreted, as calculated by this method, is approximately 1.5 micrograms per 100 gm. of body weight per 24 hours. This value is lower than that reported by Dempsey and Astwood (5.2 micrograms *l*-thyroxine per 100 gm. body weight per day[14]), but higher than that estimated by Griesbach and Purves (2.25 micrograms *dl*-thyroxine per 100 gm. body weight per day[15]). These previous estimates are based on methods which depend on the replacement of circulating thyroid hormone by injections of crystalline thyroxine.

Studies with Surviving Slices of Thyroid Tissue

The demonstration that iodine concentration and synthesis of thyroxine and diiodotyrosine can be made to take place in *surviving slices* of thyroid tissue is a most interesting outcome of the biological application of the radioactive isotope of iodine.[16] This was made possible by the sensitivity of the radioactive procedure, which is such as to permit the accurate measurement of traces of newly synthesized thyroxine and diiodotyrosine. The results provided by the use of this new tool have served to shed new light on the mechanism of the biosynthesis of thyroxine and diiodotyrosine.

Iodine-Concentrating Capacity of Thyroid Tissue Slices. A comparison of the iodine-concentrating capacity of surviving slices of thyroid gland, liver, and spleen is presented in TABLE 2. 300 mg. of each tissue was sus-

TABLE 2

IODINE CONCENTRATION BY SURVIVING LIVER, SPLEEN, AND THYROID SLICES

Tissue	*Iodide I^{127} added to Ringer's, μg.*	*Time of incubation, hours*	*Per cent of Ringer's I^* recovered in slices*	*Micrograms of Ringer's I^{127} incorporated into slices*
Sheep liver	0†	1	2.4	
Sheep thyroid	0†	1	81.4	<0.3
Beef spleen	0†	2	1.5	
Beef thyroid	0†	2	87.9	<0.3
Sheep thyroid	0†	1	86.2	<0.3
Sheep thyroid	20	1	61.3	12

† When no iodide was added, the Ringer's medium contained less than 0.3 microgram of iodine.

pended in 3 cc. of a Ringer-bicarbonate solution containing a tracer amount of radioactive iodide. That excised thyroid tissue can retain its extraordinary capacity for accumulating iodine is well brought out by the finding that as much as 80 per cent of the I* that had been added to the medium was removed by the 300 mg. of thyroid slices in one hour. In 2 hours, close to 90 per cent of the I* was found within the slice. In similar intervals, less than 5 per cent of the medium's I* was taken up by 300 mg. of liver or spleen.

The most rapid uptake of the added I* by the surviving thyroid slices occurred during the first 10 minutes (approximately 40 per cent). By the end of 30 minutes, approximately 60 per cent of the I* of the Ringer's medium had entered the slices.

In the experiments described above, no I^{127} was added to the Ringer's medium. The traces of I^{127} present in the reaction flask were due to the impurities of the reagents used in the preparation of the Ringer's solution. The addition, however, of a known quantity of iodide I^{127} to the Ringer's solution in which the thyroid slices are suspended, permitted the measurement of the absolute amounts of I^{127} that entered the thyroid slice. The results are shown in TABLE 2. Thus, approximately 12 micrograms of iodide entered the slices in one hour when 20 micrograms were added to the Ringer's medium.

The IN VITRO *Synthesis of Thyroxine and Diiodotyrosine.* When 225 mg. of surviving sheep thyroid slices were incubated for 2 hours in a Ringer's medium to which a tracer dose of radioactive iodide had been added, it was found that as much as 85 per cent of the labeled iodine was organically bound, about 75 per cent as diiodotyrosine and about 10 per cent as thyroxine (TABLE 3). The thyroid iodine was fractionated by a modification of Blau's butyl-alcohol procedure.[8]

TABLE 3

THYROXINE AND DIIODOTYROSINE FORMATION BY SHEEP THYROID TISSUE AND BY DESICCATED THYROID POWDER

Form of thyroid tissue	*Weight of preparation used, mg.*	*Volume of Ringer's solution, cc.*	*Incubation period, hours*	*Per cent of Ringer's I* recovered as*	
				Thyroxine	*Diiodotyrosine*
Slices	225	4	2	9.6	76.8
Homogenate†	225	4	2	1.5	8.7
Desiccated powder	50	4	2	0.7	7.0

† Prepared in all-glass homogenizer.

Cytochrome-Cytochrome Oxidase System Participates in the Synthesis of Thyroxine and Diiodotyrosine by Thyroid Tissue. Tissue organization (TABLE 3) was found to be of utmost importance in the *in vitro* conversion of inorganic iodide to thyroxine and diiodotyrosine.[16] Little formation of either compound was observed when tissue organization was disrupted by homogenization, a finding that suggests the participation of an intracellular enzyme system in these syntheses.

Evidence that oxidative enzymes are involved was obtained by a study of the effects of anaerobiosis and of substances that are inhibitors of cytochrome oxidase.[17] The results are shown in TABLE 4. Sulfide, azide, and cyanide in low concentrations had strong inhibitory effects on the formation of thyroxine and diiodotyrosine. Only 5–15 per cent of the normal synthesis occurred in the presence of these agents. The complete absence of oxygen or the presence of a high concentration of carbon monoxide in the atmosphere above the Ringer's solution also had marked inhibitory effects on the organification of the Ringer's I*. The inhibitory effect of the carbon monoxide was partially reversible in the presence of strong light. The inhibition of the incorporation of I* into thyroxine and diiodotyrosine by each one of these poisons, together with the marked effect of light on the inhibition by CO (which is perhaps the best single piece of evidence for this point) strongly suggests that the cytochrome-cytochrome oxidase system is involved in the formation of these iodine compounds by the thyroid gland.

TABLE 4

EFFECT OF VARIOUS INHIBITORS ON SYNTHESIS OF THYROXINE AND DIIODOTYROSINE BY SURVIVING THYROID TISSUE SLICES AS MEASURED WITH I*

Inhibitor	*Concentration in Ringer's solution or in atmosphere above solution*	*Per cent inhibition*	
		Thyroxine formation	*Diiodotyrosine formation*
RESPIRATORY INHIBITORS:			
Azide	0.005M	90	89
Cyanide	0.01M	94	83
Sulfide	0.003M	89	85
Anaerobiosis	95 per cent N_2 5 per cent CO_2	67	54
Carbon monoxide	90 per cent CO 5 per cent CO_2 5 per cent O_2	71 (in dark) 46 (in light)	81 (in dark) 39 (in light)
GOITROGENIC SUBSTANCES:			
Thiouracil	0.001M	95	90
Sulfanilamide	0.001M	47	58
p-Aminobenzoic acid	0.001M	73	79

The Effect of Goitrogenic Substances on the IN VITRO *Synthesis of Thyroxine and Diiodotyrosine.* The *in vitro* technique for measuring the conversion of radioactive inorganic iodide to thyroxine and diiodotyrosine provided a method for testing directly the action of the goitrogenic compounds on the thyroid glands.[18] As shown in TABLE 4, when surviving thyroid slices were incubated in the presence of very low concentrations of thiouracil, sulfanilamide, and *p*-aminobenzoic acid, there was a marked depression in the formation of both diiodotyrosine and thyroxine. Many other antithyroid compounds have similar inhibitory effects on this *in vitro* reaction.[19] These findings are in accord with the observations of MacKenzie and MacKenzie,[20] as well as of Astwood *et al.*,[21] who concluded from experiments on intact animals that such antithyroid compounds as thiouracil and sulfanilamide exert their effects by interfering with the synthesis of thyroid hormone.

The Question of Exchange. In the foregoing sections, it was assumed that the appearance of radioactive iodine in the organic iodine fractions of the thyroid indicates the formation of new molecules of diiodotyrosine and thyroxine. The possibility, however, that simple exchange reactions occur must be considered. Exchange, in the present instance, may be defined as the formation of radiothyroxine or radiodiiodotyrosine from radioactive inorganic iodide and pre-formed non-radioactive thyroxine or diiodotyrosine molecules. The conditions necessary to make exchange occur between molecular iodine and diiodotyrosine and between iodide ions and diiodotyrosine have been recently determined by Miller *et al.*[22] Convincing evidence, however, that the incorporation of inorganic radio-iodide into thyroxine and diiodotyrosine as measured here is *not* simply an exchange reaction, but represents the formation of new molecules of these substances, is provided by the following experimental findings:

(*1*) When surviving thyroid slices were incubated in a Ringer medium to which radioactive iodide had been added, a large part of the radio-iodine was soon incorporated into thyroxine and diiodotyrosine. Organic binding of iodine was greatly reduced, however, when tissue organization was disrupted by homogenization; in this case, nearly all of the radio-iodine was recovered in the inorganic iodine fraction (TABLE 3).

(*2*) The conversion of radioactive inorganic iodide to thyroxine and diiodotyrosine by surviving thyroid slices was greatly inhibited under anaerobic conditions or in the presence of cytochrome oxidase inhibitors. These findings strongly imply the participation of an intracellular enzyme system in the formation of these iodinated compounds.

(*3*) It has been shown by the MacKenzies and by Astwood *et al.* that goitrogenic compounds depress hormone formation by the thyroid gland. These same compounds were found to inhibit both the *in vitro* and the *in vivo* conversion of inorganic radioactive iodide to thyroxine and diiodotyrosine by thyroid tissue. A single injection of 10 mg. of thiouracil into a rat completely abolished, for some time, the thyroid's capacity of converting circulating radioactive iodide to diiodotyrosine and thyroxine.

The Mechanism of Iodine Fixation by the Thyroid Gland

It was pointed out in the first section of the paper that, as early as 15 minutes after the injection of a *tracer dose* of radio-iodide into rats, over 90 per cent of the radioactivity in the thyroid gland is organically bound. It would appear from this observation that the selective uptake of iodine by the thyroid depends on the ability of this tissue to convert iodine to diiodotyrosine and thyroxine. This mechanism, however, will not account for iodine concentration by the thyroid when relatively *large amounts* of iodine, rather than tracer doses, are injected. Thus, Leblond observed that, after the injection into guinea pigs and rats of 500 micrograms of labeled iodide per 100 gm. body weight, the largest part of the iodine fixed by the thyroid remained as inorganic iodide for some time.[23] Lein[24] reported that, after the intravenous injection of 35 micrograms of labeled iodine into a rabbit, most of the iodine fixed by the thyroid during the first 10 minutes appeared in the inorganic fraction (acetone-soluble). These findings have been confirmed in our laboratory. One hundred micrograms of labeled iodine was injected into rats and their glands removed one hour later; at this interval, approximately 50 per cent of the iodine taken up by the gland was in the inorganic fraction (trichloroacetic acid-soluble). These findings make it appear that there is some mechanism in the thyroid gland for concentrating inorganic iodine which is not dependent upon its conversion to organic iodine.

Further evidence was provided by the use of antithyroid substances. With the aid of these compounds, it was possible to separate almost completely the iodine-concentrating capacity of thyroid tissue from the mechanism for iodine organification. This was first shown in a study in which surviving thyroid slices were suspended in an oxygenated Ringer medium containing a tracer dose of I*. Although the capacity of the slices to convert the I* to organically-bound iodine was greatly depressed in the presence of 0.001M thiouracil, the latter had only slight effect on the uptake of I* by the thyroid slices.[18]

Subsequently, several investigators have demonstrated that the enlarged thyroid gland of the thiouracil-treated rat, though lacking the capacity to synthesize organic iodine compounds, retains its capacity to concentrate iodine.[25, 26] The iodine taken up under these conditions is not bound to protein and, as shown by Vanderlaan and Bissell,[27] does not remain in the gland very long. Their curves depicting the uptake of iodine by the thyroids of propylthiouracil-treated rats at various times after the injection of 50 or 500 micrograms of KI are similar in shape to that found for the uptake of iodine by plasma. It seems, therefore, that the iodine concentration observed in the goitrogen-inhibited thyroid is proportional to the iodine level in the circulating fluid. However, since the level of iodine in the gland rises to several hundred times that of the plasma, there can be little doubt that some type of selective mechanism for concentrating iodine is still operating in this inhibited gland.

It is shown in FIGURE 2 that the iodine-concentrating mechanism of the propylthiouracil-inhibited thyroid does not depend upon the injection of a large dose of iodine. Its operation is also demonstrable when a *tracer dose* of labeled iodide is injected. Under these circumstances, almost 9 per cent of the injected I* was found in the thyroid glands in one hour, whereas in 24 hours only two per cent was found there. At both

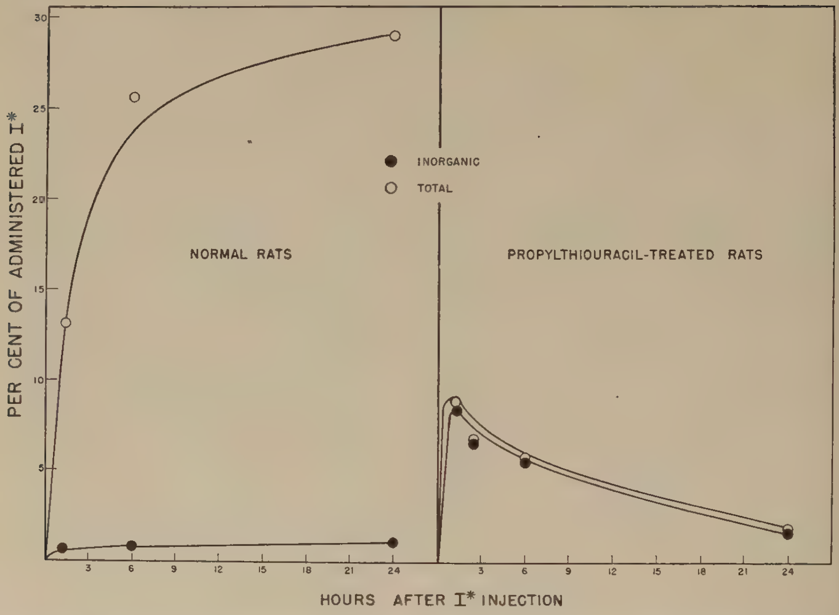

FIGURE 2. The uptake of a tracer dose of I* by the thyroids of normal and propylthiouracil-treated rats.

time intervals, practically all of the radioactive iodine was recovered in the inorganic fraction. In control rats, only about 4 per cent of the radioiodine in the thyroid was in the inorganic fraction at the one-hour interval.

When 100 micrograms of labeled iodine was injected into propylthiouracil-treated rats (FIGURE 3), over 10 per cent of it appeared in the thyroids in half an hour. Thereafter, the iodine concentration fell off rapidly, and in 26 hours had diminished almost to its original value. All of the radio-iodine in the gland was in the form of inorganic iodide. This was shown by the finding that it was readily oxidized to I_2 by excess iodate in a slightly acidic solution. None of it precipitated with the proteins upon treatment of the thyroid tissue with trichloroacetic acid, and all of it passed readily through a dialysis membrane.

From the above experiments, it appears that iodine can be initially fixed in the thyroid gland by some mechanism that is distinct from the reactions responsible for converting it to an organic form.

Circulating Iodine

Plasma Protein-Bound Iodine; Its Metabolic Significance

Although the exact nature of the circulating thyroid hormone remains to be determined, the concentration of the fraction of plasma iodine designated *protein-bound* has gained wide use as an indicator of thyroid activity in man.[28-32] This fraction of plasma iodine is the iodine

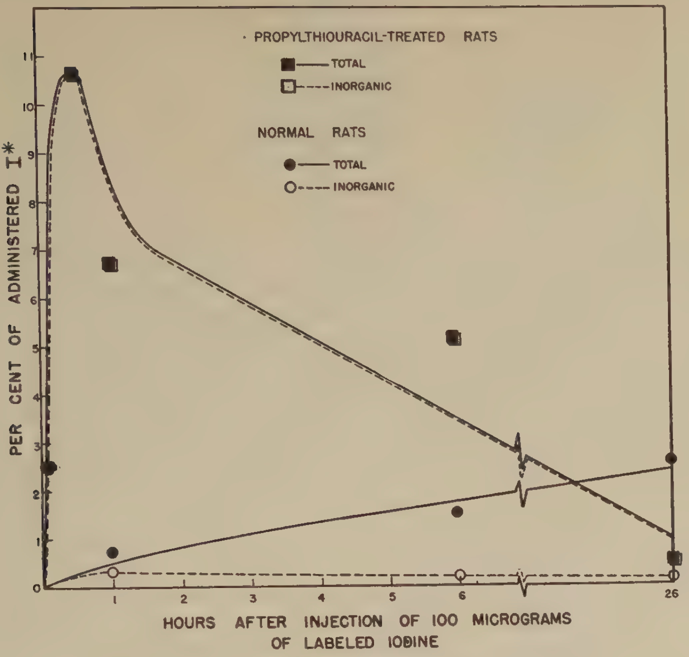

FIGURE 3. The uptake of 100 micrograms of labeled iodine by the thyroids of normal and propylthiouracil-treated rats.

which is precipitated from plasma along with its proteins by such agents as tungstic acid, zinc hydroxide, or acetic acid in the presence of heat and which, in addition, cannot be freed from these proteins by simple washing. Investigations with radioactive iodine have helped to throw light on the metabolic significance of this iodine fraction of plasma.[33] Such studies are described in this section.

Rate of Formation of Plasma Protein-Bound Iodine in Thyroidectomized Rats as Measured with Radioactive Iodine. The rate at which injected radioactive inorganic iodide is converted to protein-bound iodine of plasma is shown in TABLE 5. After its introduction into *normal* rats maintained on a low iodine intake, radioactive inorganic iodide makes its appearance rapidly in the protein-bound iodine fraction of plasma. In 13 hours, 50 per cent of the plasma I* was found to be protein-bound, in 25 hours 90 per cent.

In the *completely thyroidectomized* rat, on the other hand, only 5 to 10 per cent of the plasma I* was protein-bound as late as 25 hours after the injection (TABLE 5). These results indicate that the formation of protein-bound iodine is greatly diminished in completely thyroidectomized rats.

As was to be expected, thyroidectomy also depressed the concentration of protein-bound iodine in plasma (TABLE 6). The level of this con-

TABLE 5

FORMATION OF PLASMA PROTEIN-BOUND I* BY THYROIDECTOMIZED RATS†

Expt.	*Treatment*	*Interval when animals sacrificed*		*Plasma*		
		After operative procedure, hours	*After I* injection, hours*	*Total I* as per cent injected dose per 10 cc. plasma*	*Protein-bound I* as per cent of injected dose per 10 cc. plasma*	$\frac{\text{Protein-bound } I^*}{\text{Total } I^*} \times 100$
1	Sham Operated Control	60–72	13	1.1	0.54	49
	Sham Operated Control	60–72	13	0.96	0.65	68
	Thyroidectomized	60–72	13	3.0	0.16	5.3
	Thyroidectomized	60–72	13	2.9	0.17	5.9
	Thyroidectomized	60–72	13	2.8	0.22	7.9
2	Sham Operated Control	64–72	25	1.1	0.94	85
	Sham Operated Control	64–72	25	1.3	1.1	88
	Sham Operated Control	64–72	25	1.2	1.1	94
	Sham Operated Control	64–72	25	2.0	1.8	91
	Thyroidectomized	64–72	25	2.4	0.18	7.5
	Thyroidectomized	64–72	25	2.0	0.15	7.5
	Thyroidectomized	64–72	25	1.4	0.083	5.9

† The completeness of all thyroidectomies was checked by the radioautograph procedure.

TABLE 6

PLASMA PROTEIN-BOUND IODINE OF RATS 3 AND 16 DAYS AFTER THYROIDECTOMY

Controls, micrograms per cent	*3 days after thyroidectomy, micrograms per cent*	*16 days after thyroidectomy, micrograms per cent*
3.1	1.2	1.3
3.1	0.9	1.2
3.6	1.1	1.1

stituent fell in 3 days after thyroidectomy from 3.3 micrograms per cent (average control value) to values of about 1.0 microgram per cent. The significance of these low values, however, is difficult to assess, since they depend upon colorimetric readings which differ only slightly from those given by the reagent blanks. Whether there exists, in the completely thyroidectomized rat, a small amount of protein-bound iodine must therefore remain an open question.

Rate of Formation of Plasma Protein-Bound Iodine in Animals Injected with Thyrotropic Hormone. The rate of appearance of injected radioactive inorganic iodide in protein-bound iodine of plasma of hyperthyroid animals is shown in TABLE 7. A striking increase above control values was observed as early as 3 hours after I* injection. At this interval, the percentage of the injected I* found in the protein-bound iodine fraction per 10 cc. of plasma varied from 0.73 to 2.9 in hyperthyroid rats and from 0.17 to 0.29 in the control rats. In the former, 39–73 per cent of the plasma I* was protein-bound, whereas in the controls only 4.1–9.2 per cent was so bound. Large differences between the two groups were also evident 6 hours after the injection of the radioactive isotope; at this interval, 11–32 per cent of the plasma I* was protein-bound in the control rats, as compared with 82–87 per cent in the hyperthyroid rats. A similar increase was also observed in hyperthyroid guinea pigs.

The effect of injection of thyrotropic hormone on the level of protein-bound iodine of plasma is shown in TABLE 8. Significant increases were observed both in the rats and in the guinea pigs. In the latter, the protein-bound iodine level rose from a mean value of 2.1 micrograms per cent to one of 5.8 micrograms per cent, an increase of 175 per cent.

In summary, it may be stated that, with respect to both its concentration and—what is new—its *rate of formation*, protein-bound iodine of plasma responds to experimentally induced changes in thyroid activity in a manner to be expected in the case of a circulating thyroid hormone. Thus, not only was the level of protein-bound iodine of plasma elevated by the injection of thyrotropic hormone and depressed by the removal of the thyroids, but the rate of appearance of injected radioactive inorganic iodide in the protein fraction of plasma was greatly diminished by thyroidectomy and greatly augmented by injection of thyrotropic hormone. *This rate of appearance may prove as useful as the level of protein-bound iodine for testing thyroid activity.*

TABLE 7

FORMATION OF PLASMA PROTEIN-BOUND I* BY RATS AND GUINEA PIGS INJECTED WITH THYROTROPIC HORMONE

Animal	*Interval after I* injection, hours*	*Controls*			*Thyrotropic hormone*†		
		Weight of thyroid glands, mg.	*Protein-bound I* as per cent of injected dose per 10 cc. plasma*	$\frac{\text{Protein-bound I*}}{\text{Total I*}} \times 100$	*Weight of thyroid glands, mg.*	*Protein-bound I* as per cent of injected dose per 10 cc. plasma*	$\frac{\text{Protein-bound I*}}{\text{Total I*}} \times 10$
Rats	3	23	0.29	7.3	36	2.9	73
	3	22.5	0.21	5.2	30	1.3	39
	3	29	0.17	4.1	76	0.73	53
	3	33.5	0.26	9.2			
	6	31.5	0.44	30	39	2.0	82
	6	19	0.36	11	49	4.1	88
	6	33	0.34	27	43.5	3.1	87
	6	38	0.31	32			
Guinea pigs	16	75	0.0041	7.5	113	0.070	77
	16	56	0.0045	12	147	0.14	83
	16	68	0.0031	12	106	0.11	77
	16	68	0.0064	18	120	0.13	81
					112	0.099	86

† The rats were injected with 10 mg. of thyrotropic hormone twice daily for 4 days. The 3-hour and 6-hour groups also received one injection on the 5th day at the same time that the I* was injected. The guinea pigs were treated as described in TABLE 8.

TABLE 8

EFFECT OF THYROTROPIC HORMONE ON CONCENTRATION OF PROTEIN-BOUND IODINE IN PLASMA

Animal	*Treatment*	*Weight of thyroid glands, mg.*	*Plasma Protein-bound iodine, micrograms per cent*
Rats	Controls	33	3.2
	Controls	28	3.2
Rats	3 injections with thyrotropic hormone over 24-hour period, each injection 10 mg.†	34	4.9
		45	6.2
		43	5.3
		43	5.7
Guinea pigs	Controls	75	2.2
	Controls	56	2.0
Guinea pigs	2 mg. thyrotropic hormone injected once daily for 3 days and twice on 4th day. Animals sacrificed morning of 5th day.	113	6.1
		147	6.1
		106	4.4
		120	6.8

† The thyrotropic hormone preparation contained 4 guinea pig units per mg.

The Thyroxine-like Fraction of Plasma

The small amount of iodine in plasma makes difficult its exact chemical identification. Although values for the thyroxine content of blood have been reported,[34] their accuracy must remain doubtful until confirmation is provided by other investigators.

In contrast to the chemical measurement of thyroxine, determinations of *radiothyroxine* need not suffer any loss in accuracy when this iodine fraction is present in very minute quantities. The accuracy of the separation, when only radioactive measurements are involved, can be maintained by the addition of non-radioactive carriers. Thus, in the solution of certain problems, the judicious use of the radioactive isotope of iodine has circumvented the technical difficulties inherent in the chemical measurement of very minute concentrations of iodine.

In a study carried out in this laboratory by Morton *et al.*, plasma was removed from normal rats at intervals after the injection of radioiodide.[35] The plasma was hydrolyzed with 2N NaOH and, after the addition of non-radioactive thyroxine, diiodotyrosine, and inorganic iodide as carriers, was subjected to a butyl-alcohol fractionation. The radioactivity was separated into the following 3 fractions by the procedure previously outlined: thyroxine-like, diiodotyrosine-like, and inorganic.

The curves in FIGURE 4 show the distribution of the plasma radioactive iodine among these 3 fractions. A significant fact emerging from this experiment is that, as early as 24 hours after the injection, 80 per cent of the radioactive iodine of plasma is contained in the thyroxine-like fraction.

For a different group of rats, the rate of appearance of the injected

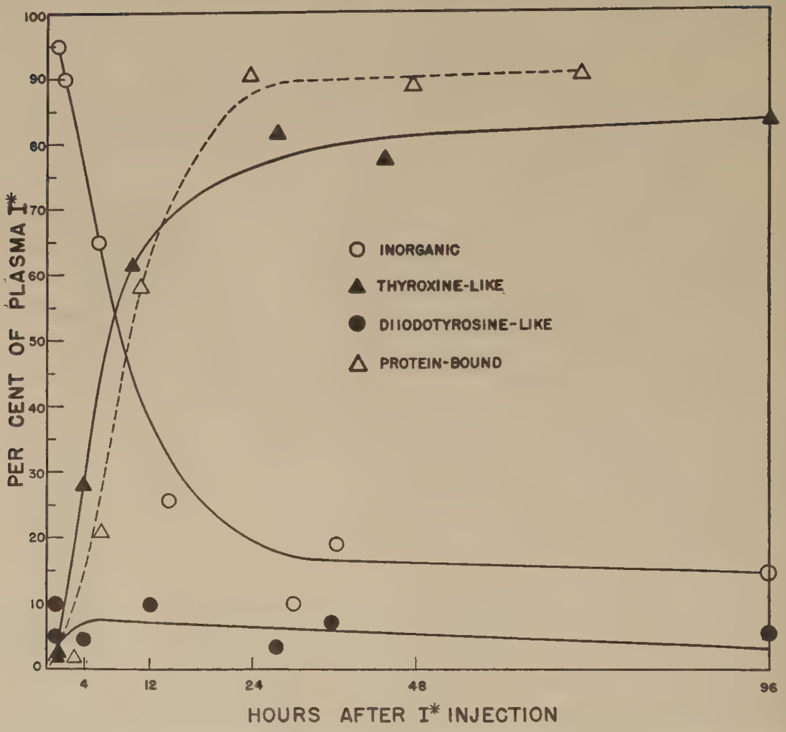

FIGURE 4. The distribution of plasma I* among the various iodine fractions. For explanation, see text.

radio-iodide in the protein-bound iodine fraction of plasma has also been plotted on the same axes (FIGURE 4). The resemblance between the curves for protein-bound iodine and thyroxine helps to throw light on the nature of hormonal iodine. The similarity in the rates of incorporation of radio-iodine in these two fractions suggests that protein-bound iodine of plasma is largely composed of some thyroxine-like iodine compound attached to protein. The finding that much or all of the protein-bound iodine of plasma is extractable with organic solvents also supports the view that the circulating thyroid hormone is in the form of a small thyroxine-like molecule loosely attached to protein.[36, 37]

It is interesting to note that, in the experiments described above, only 5–10 per cent of the plasma radioactivity was found in the diiodotyrosine fraction at all intervals up to 96 hours after the injection. Since, in the thyroid gland, the amount of newly formed radiodiiodotyrosine exceeds that of newly formed radiothyroxine, this finding demonstrates a preferential output of thyroxine into plasma by the gland.

The Role of the Anterior Pituitary in Iodine Metabolism

Much has been gleaned from morphological studies on the nature of the hypophyseal control of thyroid function, but an understanding of the relation of thyrotropic hormone to iodine metabolism and thyroxine synthesis by the thyroid gland has had to await the introduction of radioactive iodine as a labeling agent and the development of micromethods for the measurement of iodine and thyroxine. The recent work made possible by these new tools is reviewed below.

The Hypophysectomized Animal

The Iodine Content of Gland and Plasma. The effects of hypophysectomy upon the concentration of iodine in plasma and thyroid gland of the rat are shown in TABLE 9. A decrease in the level of plasma iodine was observed as early as 2 days after hypophysectomy, and by 4 days a decrease of about 50 per cent had occurred in both total and protein-bound iodine of plasma. No further significant drop was observed in rats examined 44 days after hypophysectomy.

TABLE 9

EFFECT OF HYPOPHYSECTOMY ON THYROID AND PLASMA IODINE

	Days after hypophysectomy	*Thyroid weight per 100 gm. body weight, mg.*	*Thyroid iodine*				*Plasma iodine, μg. per cent*	
			Thyroxine		*Total*			
			Amount, μg.	*Concentration, mg. per cent*	*Amount, μg.*	*Concentration, mg. per cent*	*Protein-bound*	*Total*
Control	2	13.0	2.3	6.6	6.8	19	2.7	
Operated		10.0	2.2	8.5	6.8	26	2.3	
Control	4	13.5	2.7	8.4	8.5	26	2.6	2.5
Operated		11.1	3.5	13	11.9	45	1.2	1.2
Control	9	10.9	1.9	7.2	6.9	26	2.6	2.7
Operated		7.5	2.2	13	8.6	51	1.1	1.5
Control	44	10.1	2.8	12	9.0	38	3.5	
Operated		6.7	3.1	23	10.2	76	1.9	
Operated	1 yr.	8.6	2.3	15	10.0	63	1.9	

A decrease in the weight of the thyroid gland was detected as early as 2 days after hypophysectomy and was quite pronounced in 9 days. However, at no time up to one year after hypophysectomy was a decrease observed in the amount of thyroxine or of total iodine contained in the gland (TABLE 9). This resulted in a greatly increased concentration of iodine in the gland of the hypophysectomized rat; thus, in 44 days, the thyroid glands of the operated and control rats contained, respectively, 0.076 and 0.038 per cent total iodine and 0.023 and 0.012 per cent thyroxine iodine.

The observation that the level of protein-bound iodine in plasma falls as early as 48 hours after excision of the pituitary gland is not surprising in view of the well-known effects of hypophysectomy upon the morphology of the thyroid gland. The presence, however, of normal and even greater than normal amounts of thyroxine in the gland during the time that this is occurring, throws new light on the mechanism by which thyroxine is released into the circulation by the gland. These findings leave no doubt that a lowered thyroxine content of plasma is not a *direct* stimulus to the thyroid gland to release its thyroxine. They demonstrate quite clearly that, even in the gland that contains an amount of thyroxine greater than normal, its release into the bloodstream requires the action of the thyrotropic hormone.

Studies with the Radioactive Isotope of Iodine. The data presented above may be considered to provide a picture of the steady state of iodine metabolism in the hypophysectomized rat. The conclusions derived from such data have been supplemented considerably by the dynamic picture obtained with the use of radioactive iodine.[35]

Tracer doses of I* were injected into hypophysectomized and normal rats and the fate of this iodine compared. The injection of a tracer dose of I* (*i.e.*, carrier-free) does not increase the iodine content of the animal. At an early hour, a much smaller amount of the *initially labeled iodide* appeared in the thyroid gland of the hypophysectomized than in that of the normal rat (FIGURE 5). Thus, in 4 hours, the normal thyroids contained approximately 60 per cent of the injected I*, while at the same time-interval the gland of the hypophysectomized rat contained less than 5 per cent. Moreover, the character of the curves depicting the uptake of the labeled iodine differed in the two types of rats. The gland of the hypophysectomized rat should not be regarded as completely inactive, however, for it showed a continuous rise in its content of the injected I* during the entire period of observation and at 96 hours held about 25 per cent of the administered I*.

A striking difference between normal and hypophysectomized rats was also observed in the manner in which injected I* disappeared from the plasma (FIGURE 5). The I* left the blood more rapidly in the former, in keeping with the more rapid uptake of I* by the thyroids. The rise in the I* content of the plasma of normal rats after 12 hours (not observed in the hypophysectomized rats) reflects the release of labeled hormone from the thyroids.

In order to determine the fate of the I* that enters the gland, this iodine was separated into three fractions: diiodotyrosine, thyroxine, and inorganic iodide. The distribution of the I* of each gland among these three fractions is shown in FIGURE 6. The rapidity with which iodine that enters the *normal* gland is organically bound is again clearly brought out. At the 4-hour interval, about 90 per cent of the I* contained in the normal gland is organically bound, most of it as diiodotyrosine and about 20 per cent as thyroxine. At later intervals (up to 96 hours), the percentage in the form of thyroxine increased, while that in the form of diiodotyrosine decreased.

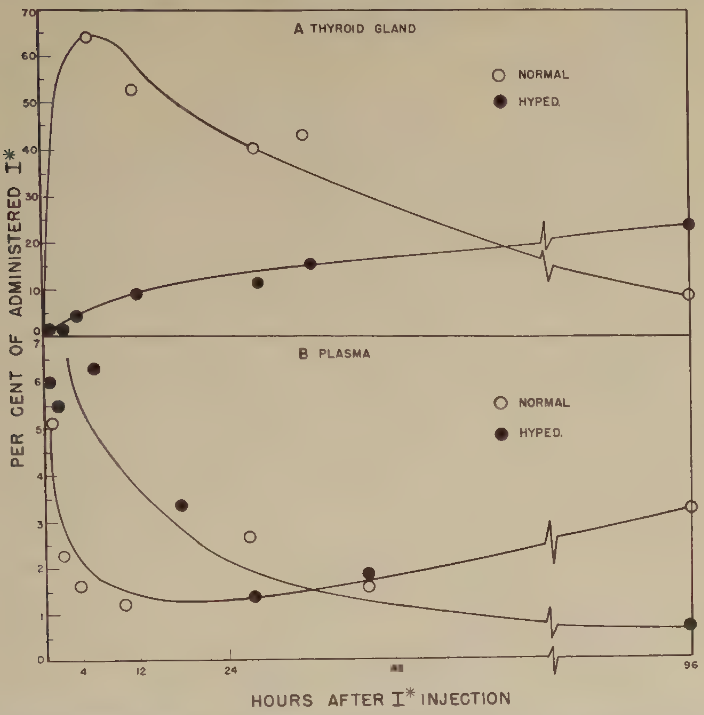

FIGURE 5. The uptake of total I* by the whole thyroid gland (*A*) and by entire plasma (*B*) of the normal and hypophysectomized rat. Entire plasma was estimated as 5 per cent of the total body weight. A tracer dose of I* was injected intraperitoneally into each rat.

FIGURE 6 shows that whatever iodine does enter the thyroid gland of the hypophysectomized animal is rapidly converted to diiodotyrosine. About 80 per cent of the gland's I* was present as diiodotyrosine as early as 4 hours, but (interestingly enough) the same percentage of the gland's radioactive iodine was present as diiodotyrosine as late as 96 hours after the injection of the isotopic iodine. The conversion of diiodotyrosine to thyroxine appears to be interfered with in the absence of hypophysis. This interpretation of the data is fully supported by the finding (FIGURE 7) that practically none of the plasma I* of the hypophysectomized rat appears as thyroxine iodine even as late as 96 hours, a time when approximately 80 per cent of the I* contained in plasma of the normal rat is thyroxine iodine (FIGURE 4).

The Animal Treated with Thyrotropic Hormone

The Iodine Content of Gland and Plasma. Many workers have reported a decreased iodine content in the thyroid gland of animals treated with preparations of thyrotropic hormone.[38-43] Closs, Loeb, and MacKay[40] found an increase in blood iodine as well as a decrease in gland iodine in

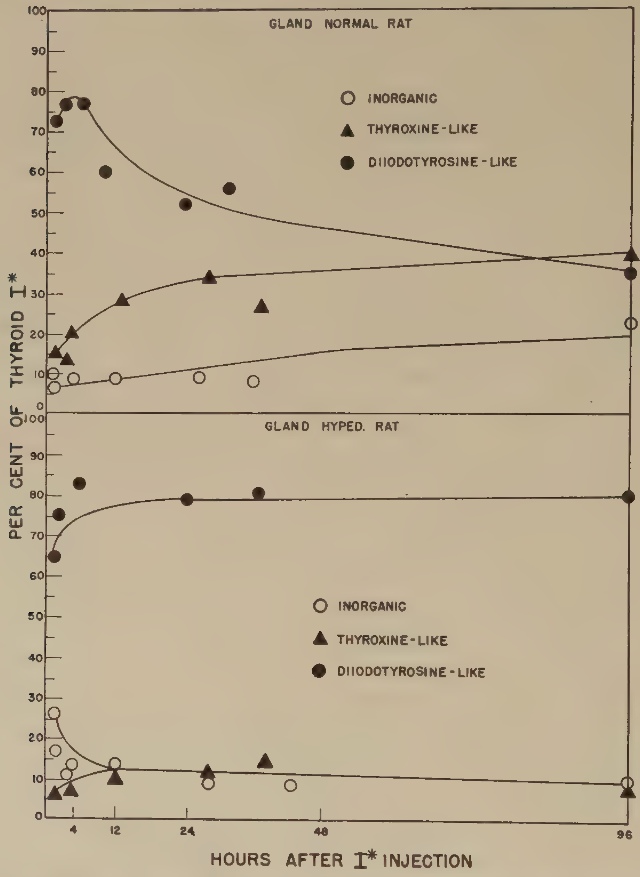

FIGURE 6. The distribution of thyroid I* in normal and hypophysectomized rats. Each rat received intraperitoneally a tracer dose of I*. For explanation, see text.

guinea pigs injected with a pituitary extract. These findings have been confirmed in this laboratory (TABLE 10, TABLE 8). The data in TABLE 10 show that the decrease in gland iodine results from a fall in the amount of organically-bound iodine (thyroxine and diiodotyrosine) but not of inorganic iodide.

Studies with Radioactive Iodine—IODINE-CONCENTRATING CAPACITY. The thyroid glands of animals injected with thyrotropic hormone have a greater than normal capacity for fixing administered I*. This has been shown to be the case whether the radioactive iodine was administered as a tracer dose (*i.e.*, carrier-free) or along with carrier. Such increases in uptake have been found in the guinea pig,[44–46] the rabbit,[47] and the rat.[37]

A study by Morton *et al.*[46] revealed that, whereas 4–9 per cent of an injected tracer dose of I* was present in the thyroid glands of normal

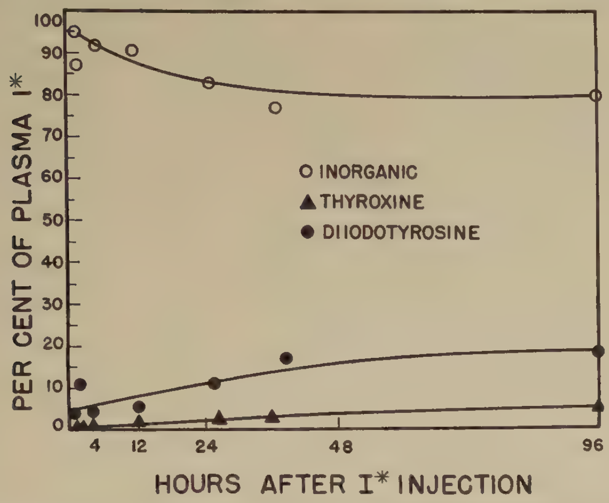

FIGURE 7. The distribution of plasma I* in the hypophysectomized rat. For explanation, see text.

TABLE 10

THE EFFECT OF THYROTROPIC HORMONE ON THE IODINE (I^{127}) FRACTIONS OF THE THYROID GLAND OF THE GUINEA PIG

Weight of gland	*Inorganic fraction*			*Diiodotyrosine fraction*			*Thyroxine fraction*		
	Amount, µg.	*Concentration, mg. per cent*	*Per cent of total iodine*	*Amount, µg.*	*Concentration, mg. per cent*	*Per cent of total iodine*	*Amount, µg.*	*Concentration, mg. per cent*	*Per cent of total iodine*
Controls									
75	0.47	0.6	1.3	28.0	37	75	9.0	12	24
56	0.27	0.5	1.1	19.3	34	76	5.8	10	23
68	0.13	0.2	0.4	23.2	34	74	8.2	12	26
68	0.26	0.4	1.2	16.5	24	78	4.3	6.3	20
Hyperthyroid†									
113	1.0	0.9	5.0	14.8	13	74	4.2	3.7	21
147	0.76	0.5	6.3	8.9	6.1	74	2.3	1.6	19
106	0.45	0.4	7.6	4.3	4.1	72	1.2	1.1	20
120	0.80	0.7	6.8	8.6	7.2	73	2.4	2.0	20
112	0.82	0.7	5.2	11.1	10	70	3.9	3.5	25

† Injected with thyrotropic hormone as described in TABLE 8.

guinea pigs 2 hours after the injection, 12–23 per cent was found in the hyperplastic glands of pigs treated with thyrotropic hormone. At the 26-hour interval after the injection of I*, 14–27 per cent of the administered labeled iodine was present in the normal thyroids and 32–41 per cent in the hyperactive glands. The increased uptake was apparent in all three iodine fractions of the thyroid gland. Similar results were obtained in more recent experiments shown in FIGURE 8.

THE DISTRIBUTION OF THE I* IN THE THYROID GLAND AS THYROXINE AND DIIODOTYROSINE. In the hyperthyroid animal, as in the normal, the I*, when administered as a tracer dose, is taken up by the thyroid gland

and rapidly converted there to organic iodine. The distribution of the I* within the gland among the 3 fractions, iodide, thyroxine-like, and diiodotyrosine-like, is shown graphically in FIGURE 8. Under the influence of the thyrotropic hormone, the normal distribution of the gland's I* between thyroxine and diiodotyrosine is changed. In the hyperactive gland, a smaller percentage than normal of the gland's I* was found as diiodotyrosine, whereas a larger percentage than normal was present as thyroxine.

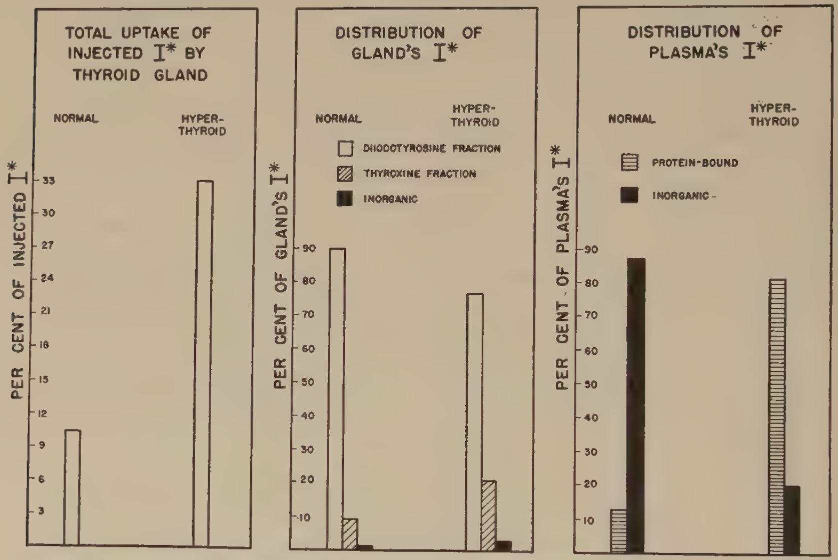

FIGURE 8. The uptake of I* and its distribution in the thyroid and plasma of normal and thyrotropic hormone-injected guinea pigs. The measurements were made 16 hours after the intraperitoneal injection of a tracer dose of the I*.

THE DISTRIBUTION OF I* BETWEEN THYROXINE AND DIIODOTYROSINE IN PERIPHERAL TISSUES. The striking effect of thyrotropic hormone on the metabolic fate of circulating inorganic iodide is shown in FIGURE 9, which depicts the distribution of the radioactive iodine contained in one of the peripheral tissues (in this case the small intestine) of guinea pigs at various time intervals after the injection of a tracer dose of radioactive iodine. It is assumed here that the movement of radio-iodine in and out of the tissues merely reflects the changes in radio-iodine concentration of the plasma.

Radioactive inorganic iodide disappeared more rapidly from the small intestine of the hyperactive than of the normal animal, a finding in keeping with the increased iodine-concentrating capacity of the thyroid gland of the thyrotropic hormone-injected guinea pig. This sharper drop in the inorganic iodide was accompanied by a sharper rise in the thyroxine-like I*.

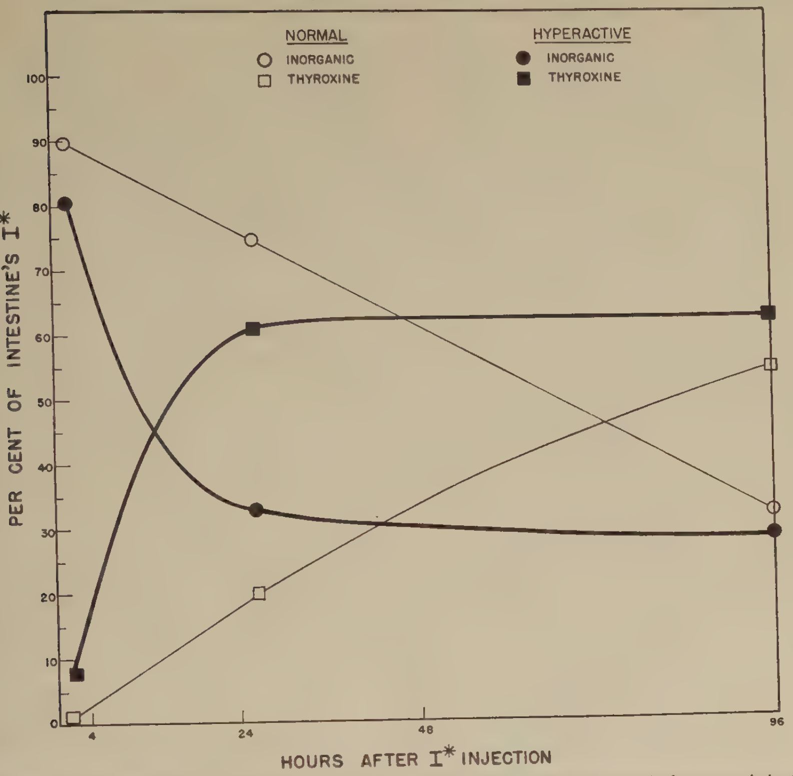

FIGURE 9. The distribution of the small intestine I* in normal and thyrotropic hormone-injected guinea pigs.

It may be concluded from the experiments reported in this section that, in the thyrotropic hormone-treated animal, there is a more rapid removal of circulating radio-iodide by the thyroid gland, a more rapid conversion of this to thyroxine, and a more rapid release of radio-thyroxine by the gland into plasma and peripheral tissues.

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Discussion of the Paper

Dr. Wilbur H. Miller (*Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company, Stamford, Connecticut*): My remarks are concerned with the question of the exchange reaction mentioned by Dr. Chaikoff. Some of us who have worked with diiodotyrosine have noticed the fact that this compound, under certain conditions, is quite unstable and can give off iodine in appreciable quantities. A somewhat detailed study of this problem has been published by Harington and Pitt Rivers.[1] Impetus for this study was furnished by the discovery of von Mutzenbecher[2] that diiodotyrosine on incubation in alkaline solution spontaneously gave rise to small quantities of thyroxine. This observation was confirmed by Block,[3] starting with synthetic diiodotyrosine. A theory for this reaction was proposed by Johnson and Tewkesbury,[4] who postulated hypoidite as the effective oxidizing agent. All of these observations point to the instability of this thyroxine precursor.

We have published[5] that diiodotyrosine undergoes an exchange reaction and, in view of such observations as above indicated, this is not too surprising. We divided the exchange behavior of diiodotyrosine into two parts: (*1*) the reactivity in the presence of iodine and (*2*) the reactivity in the presence of iodide.

In the presence of iodine in aqueous solution, the radioactivity passes either from the diiodotyrosine to the iodine, or *vice versa*, depending on the mol ratio of reactants and the compound with which the radioactive iodine atoms were associated originally. This reaction occurs readily at room temperature and the rate does vary with pH. We first observed this reaction when a thyroid tissue hydrolysate containing radio-diiodotyrosine was treated with potassium iodide-potassium iodate at around pH 2.

The exchange reaction between iodide and diiodotyrosine occurs much more slowly at room temperature, and to get a rate which is readily measured it is best to warm the reaction mixture. This exchange reaction is inhibited by compounds which react readily with iodine, such as thiouracil[6] and thiosulfate and is speeded up by very small quantities of elementary iodine. It is likely that the exchange between iodide and diiodotyrosine occurs only after some of the latter compound has decomposed with the liberation of sufficient iodine to allow this exchange to proceed. Heating would speed up both the decomposition rate and the exchange rate of diiodotyrosine.

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These observations are offered merely to indicate again the need to insure that the results of any *in vivo* work are not modified by subsequent *in vitro* procedures during which such exchanges as have been observed above might occur.

Dr. C. P. Leblond (*McGill University, Montreal, Canada*):

Dr. Chaikoff gave credit to Mann, Warren, and myself for publishing the first positive proof that diiodotyrosine was the precursor of thyroxine. The arguments, which we obtained from a study of the specific activity in the thyroids of dogs after administration of radio-iodine, were as follows:

(*1*) The specific activity of the thyroxine fraction, 48 hours after injection of radio-iodine, was greater than that of the iodide fraction at $\frac{1}{2}$, 8, or 48 hours and smaller than that of the diiodotyrosine fraction at these same time intervals. Consequently, diiodotyrosine was the only possible supplier of radio-iodine to thyroxine.

(*2*) This conclusion was in keeping with the gradual rise of the specific activity of thyroxine during the 48 hours after the injection, while the specific activity of diiodotyrosine remained steady throughout the whole period.

These results agree with the known facts of thyroxine chemistry. However, they were obtained with a small number of animals. Drs. Chaikoff and Taurog worked with a large number of animals and, by using Zilversmit's method for the analysis of the results, removed all possible doubt that Harington's theory on the synthesis of thyroxine from diiodotyrosine is correct.

IODINE ABSORPTION AND UTILIZATION UNDER THE INFLUENCE OF CERTAIN GOITROGENS

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ABSORPTION of iodine by the thyroid gland, synthetic conversion of iodine into the various chemical forms in which it occurs there, and discharge of iodine from the gland involve mechanisms varying to a greater or lesser degree according to the extent to which other mechanisms are altered. The influence of dietary limitation of iodine intake on thyroid function has been investigated experimentally and is encountered often in clinical medicine. The effects of high iodine intake have been studied to a lesser extent, and it is the purpose of this communication to present experimental data bearing on this phase of thyroid function.

Experiments by Rawson (1944) and by Chaikoff (1944) and their respective co-workers, using a radio-iodine technique, have shown that administration of thiouracil induces an effective block to the uptake of iodine by the thyroid of the rat. Franklin, Chaikoff, and Lerner (1944), however, have demonstrated that surviving sheep thyroids do absorb iodide from a Ringer-bicarbonate medium containing thiouracil, although little or none of the absorbed iodine is converted into diiodotyrosine or thyroxine, as is the case in control experiments in which thiouracil is absent from the medium (Morton and Chaikoff, 1943). Differences between iodine-absorbing capacity of thyroids of intact thiouracil-treated animals and surviving thyroids respiring in a thiouracil-containing medium are not explained on the basis of iodine concentration in the environment.

We had observed, in our experiments dealing with quantitative evaluation of antithyroid activities of various chemical substances in rats, that the level of iodine intake during the experimental period influences not only the extent to which thyroid iodine concentration is reduced but the degree of hypertrophy of the gland as well. Astwood has reported that rats are more sensitive to thiouracil if maintained on low iodine intakes. He has demonstrated, furthermore (1944–45), that iodine is absorbed by the thyroid of the thiouracil-treated rat in amounts proportional to the daily intake. Vanderlaan and Bissell (1946) showed that such absorption occurs very rapidly in the chick as well as in the rat.

With these observations in mind, we have investigated quantitatively the amount of iodine absorbed when relatively large amounts of iodide

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* The author wishes to acknowledge his thanks and appreciation for the technical assistance of Mary L. Wilson and Virginia E. Hinchman.

were given to rats receiving thiouracil and other goitrogens. We became interested, at the same time, in the form in which iodine accumulated in the gland and whether or not the goitrogenic action of antithyroid compounds was modified with iodide administration.

Procedure

Twenty-six- to twenty-eight-day female rats, weighing approximately 45 gm. at the start of the experiment, were used throughout. These were maintained at 25° C. in screen-bottom cages arranged for accurate recording of food and water intake.

Thiouracil and other antithyroid compounds were administered in a standard breeder ration containing yellow corn meal 33 parts, whole-wheat flour 31, whole-milk powder 21, linseed meal 7, brewer's yeast (Anheuser-Busch G) 3, alfalfa meal 2, dried hog liver 2, $CaCO_3$ 0.5, and C.P. NaCl 0.5. The daily intake of iodine from this diet varied between 0.7 and 1.4 micrograms, depending on the amount of food eaten. Additional iodine was administered as KI dissolved in the drinking water, which itself was iodine-free.

At the end of the experimental period, groups of 3–10 rats, similarly treated, were killed with chloroform, their thyroids removed, pooled, and weighed. They were analyzed for total iodine after alkali fusion by the method of Astwood and Bissell (1944).

In the thyroid fractionation experiments, no attempt was made to determine inorganic, thyroxine, and diiodotyrosine iodine according to current procedures. However, glands were fractionated with $Zn(OH)_2$, the precipitate being considered as protein-bound iodine and the supernatant as non-protein-bound iodine. Pooled glands were homogenized in a mortar with distilled water, transferred to a 15-cc. centrifuge tube and made up to 4–5 cc. volume. After addition of 0.25 cc. each of $Zn(SO_4)_2$ and NaOH solutions (Somogyi), the contents of the tubes were mixed and centrifuged. The precipitate was washed three times with 3–4 cc. portions of filtered $Zn(OH)_2$ solution and analyzed for iodine. The supernatant from the centrifuged $Zn(OH)_2$ precipitate and washings were analyzed separately.

Results and Discussion

Figure 1 shows the effect of increasing intakes of iodide on total thyroid iodine concentration and on thyroid weight of rats receiving 4–5 mg. thiouracil daily for a period of 10 days. With increasing intakes, iodine content of the thyroid ranged from 0–2 mg. per cent when the daily iodine intake was 8–10 micrograms, to values of over 40 mg. per cent when 10 milligrams of KI were consumed daily. At the end of the 10-day period, total iodine of the gland at the 10-microgram KI intake level was .10–.15 μg., whereas at the high iodide intake of 10 milligrams, thyroid iodine reached 4–6 μg. per rat. That this uptake was not due to simple diffusion of iodide into the thyroid from abnormally high blood

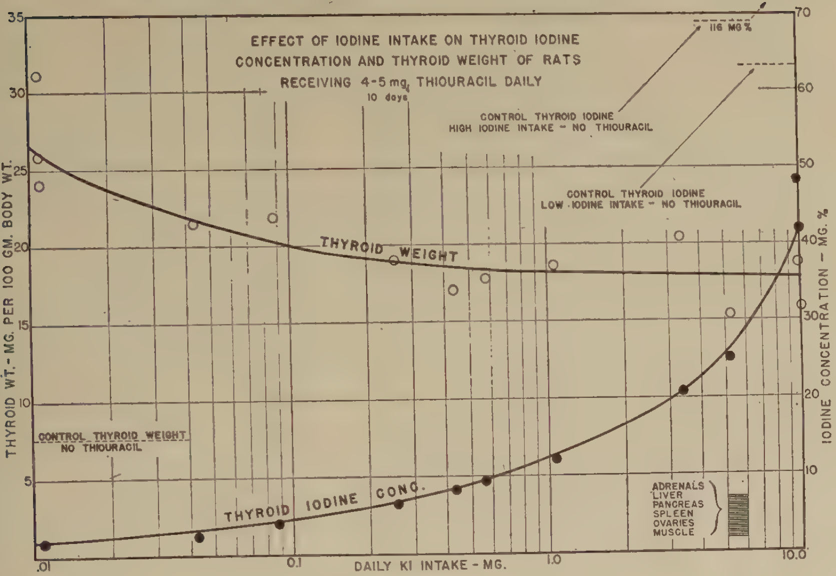

FIGURE 1

concentration is shown by the relatively low level of iodine in several other tissues at KI intake of 5–6 mg. daily. Attention is called to the levels of thyroid iodine concentration at low and high iodine intakes in control groups receiving no thiouracil in their diet.

The goitrogenic action of thiouracil is appreciably reduced with high iodine intakes. This is not so pronounced in the 10-day experiments as in those in which treatment was carried out for longer periods. Antagonism of iodide toward this effect of thiouracil is not due to a general toxic action, since growth rate of rats on thiouracil alone, iodide alone or a combination of the two in dosages employed was similar over the experimental period to that of untreated controls.

When thyroid glands from rats treated with thiouracil and iodide were fractionated with $Zn(OH)_2$, nearly all of the iodine was found in supernatants indicating its non-protein-bound character. This is illustrated in TABLE 1, in another experiment in which two groups of thiouracil-treated rats were given 10–11 micrograms and 9–10 milligrams of iodine respectively, in the drinking water. The bulk of the iodine was found in the non-protein-bound fraction, in contrast to the corresponding control group receiving no thiouracil, in which most of the iodine remained in the protein-bound fraction. It is quite evident, from these results, that the primary effect of thiouracil in causing loss of protein-bound iodine from the thyroid is not interfered with by iodide administration, and that iodine fixation in the gland with high intakes involves an independent mechanism.

These experiments were repeated in rats receiving maximal doses of

TABLE 1

THIOURACIL-IODINE ADMINISTRATION

(10 rats per group—10 days treatment)

Diet supplement	*Av. body wt. gain, gm. per day*	*Av. daily iodine intake per 100-gm. rat*	*Thyroid wt. mg./100-gm. rat*	*Thyroid iodine mg. %*	
				$Zn(OH)_2$ Ppt.	*$Zn(OH)_2$ Supt.*
.1% Thiouracil + 1 mg. KI/liter H_2O	3.2	11 μg.	30.8	0.1	0.3
.1% Thiouracil + 1000 mg. KI/liter H_2O	2.9	10 mg.	22.7	0.4	37.4
Control + 1 mg. KI/liter H_2O	4.0	10 μg.	6.7	62.2	1.4
Control + 1000 mg. KI/liter H_2O	3.4	9.7 mg.	7.2	95.6	3.0

thiouracil and other goitrogens for 25 days, followed by a similar period of combined treatment with goitrogen and high iodine. This was done in order to observe the effect of high iodine intake on iodine content of the thyroid and on goitrogenic action of the drug subsequent to depletion of iodine content by preliminary treatment with antithyroid compound.

Results are shown in TABLE 2. After a 25-day period of treatment with thiouracil, 5-aminothiadiazole-2-thiol (TC–68), 3-(phenylaminomethyl)-thiazolidine-2-thione (TC–105), KSCN, and propyl thiouracil, when the iodine content of the thyroid has been reduced to minimal levels, high iodine intake causes a reaccumulation of iodine in the gland. This is found, however, in the $Zn(OH)_2$-soluble fraction, as was the case in those experiments in which antithyroid compound and iodide were given together at the beginning of the experiment. It will be observed, also, that there occurred a significant reduction in thyroid weight in three instances after the initial treatment with antithyroid substance, and that, in the other instance, no further increase occurred when iodine was added. Again, examination of data on body weight change, or food and water consumption, indicated that this antigoitrogenic effect of iodide was not due to any toxic effect *per se.*

KSCN at a concentration of .2% in the diet caused a reduction of thyroid iodine content to 9.3 mg. per cent after 51 days on the low iodine intake of 1.4 μg. daily. Most of the iodine left in the gland was in the $Zn(OH)_2$ precipitate fraction, and only a slight enlargement of the thyroid occurred. Introduction of substantial amounts of iodine to the diet at the end of 25 days caused restoration of iodine to the thyroid and disappearance of goiter. In contrast to the blocking action of thiouracil and other potent goitrogens, KSCN is quite unable to prevent synthesis of protein-bound iodine when sufficient iodine is taken in the diet. It may be observed, however, that substantially more non-protein-bound

TABLE 2

Distribution of Iodine in Thyroid and Thyroid Weights of Rats Given a Low Iodine Diet Containing Thiouracil and Other Goitrogens for 25 Days, Followed by a 26-Day Period during which the Goitrogens were Administered Simultaneously with a High Iodine Intake. 3–6 Rats per Group. Thyroid Weight and Iodine Concentrations from Pooled Thyroids of Each Group

Exp. No.	*Goitrogen*	*Conc. in food*	*Body wt. gain gm./day*	*Av. daily iodine intake per 100-gm. rat*	*Thyroid wt. mg./100-gm. rat*	*Thyroid iodine mg. %*		*Duration of experiment, days*
						$Zn(OH)_2$ Ppt.	*$Zn(OH)_2$ Supt.*	
1422	Thiouracil	.1%	2.2	1.1 μg.	51.9	0.0	0.1	25
1252			1.4	1.0 μg.	70.2	0.6	1.6	25+26
1253			1.6	8.3 mg.	36.9	1.6	55.0	
1423	TC–68*	.1%	2.2	1.1 μg.	59.2	0.0	0.0	25
1260			1.5	1.1 μg.	67.0	1.1	0.7	25+26
1261			1.6	12.8 mg.	38.4	1.0	45.6	
1424	TC–105†	.1%	2.1	0.8 μg.	57.1	0.0	0.0	25
1267			1.4	0.9 μg.	74.5	0.6	1.4	25+26
1268			1.3	15.7 mg.	57.0	0.8	100.5	
1511–2	6-n-Propyl thiouracil	.03%	2.4	1.1 μg.	60.4	0.3	0.7	25
1523–4			1.4	1.1 μg.	93.1	0.3	2.0	25+26
1525–6			1.4	10.5 mg.	49.2	0.6	43.0	
1274	KSCN	.2%	2.1	1.4 μg.	9.3	7.2	2.1	25+26
1275			2.0	10.0 mg.	7.7	127.3	11.8	
1513–4	Controls		3.1	1.4 μg.	8.2	58.2	4.2	25
1527–8			2.5	1.4 μg.	6.9	30.8	2.8	25+26
1529–30			2.6	11.3 mg.	7.4	115.0	5.2	

* 5-Aminothiadiazole-2-thiol.
† 3-(Phenylaminomethyl)-thiazolidine-2-thione.

iodine was found in the KSCN-treated rats receiving high iodine than in the corresponding control group. These results, as a whole, are consistent with those of other investigators who have found that KSCN is goitrogenic only when iodine intake is below certain minimal levels.

The rats treated with effective antithyroid compounds gained less weight than did the controls. The decrease in weight gain was not evident until after two to three weeks of treatment with the drug. Therefore, we would contribute this decreased weight gain to the development of an athyreotic state rather than to a toxic effect of the drug given.

We also find that recovery from the effects of thiouracil and other goitrogens is accelerated by a high iodine intake. This is shown in a limited experiment in which rats were treated for 10 days with a low iodine diet containing .1 per cent thiouracil followed by a 14-day period during which no thiouracil was given and iodine intake was increased. The degree of restoration of protein-bound iodine and recovery from goiter is shown in TABLE 3. It may be noted that even at a low intake of 1.4 μg. iodine daily some absorption and fixation of iodine into the thyroid occurred and considerable reduction in thyroid weight took place. It appears that avidity of the gland for iodine increases as its content diminishes.

The effect of diiodotyrosine administration on the action of thiouracil is shown in TABLES 4 and 5. In the first experiment, diiodotyrosine at a concentration of .1 per cent in the food was administered simultaneously with thiouracil for a period of 10 days. The results are similar to those obtained with iodide at corresponding iodine intakes. Since no attempt was made to determine the diiodotyrosine content of the thyroid glands at the end of the experiment, one cannot state with certainty whether accumulation of iodine in the supernatant fraction under thiouracil treatment was diiodotyrosine itself, which is soluble in $Zn(OH)_2$, or iodide which formed by decomposition of diiodotyrosine. There is no evidence that diiodotyrosine decomposition did not occur. The antigoitrogenic effect of diiodotyrosine is similar to that obtained with iodide at corresponding intakes. Dempsey and Astwood (1943) have suggested that the antigoitrogenic action of diiodotyrosine may be due to traces of thyroxine in the compound or formed from it by oxidation. We have found that diiodotyrosine administered in the drinking water is much more antigoitrogenic than when administered in the food, particularly when solutions were prepared at infrequent intervals. These results lend support to the view that thyroxine is formed under these circumstances. In the next experiment, diiodotyrosine was incorporated into the diet subsequent to a 10-day period of thiouracil treatment. Results are shown in TABLE 5. When thiouracil is continued for 14 days after the preliminary 10-day treatment, diiodotyrosine or iodide accumulates in the non-protein-bound fraction. If thiouracil is discontinued and diiodotyrosine administered for 14 days, iodine is found mainly in the $Zn(OH)_2$ precipitate fraction. Here, again, uncertainty exists as to whether diiodo-

Table 3

Distribution of Iodine in Thyroid and Thyroid Weights of Rats Given a Low Iodine Diet Containing Thiouracil for 10 Days, Followed by a 14-Day Period during which Thiouracil is Discontinued and a High Iodine Substituted for the Low Iodine Intake. Six Rats per Group. Thyroid Weight and Iodine Concentrations from Pooled Thyroids of Each Group

Exp. No.	*Goitrogen*	*Conc. in food*	*Body wt. gain, gm.*	*Av. daily iodine intake per 100-gm. rat*	*Thyroid wt. mg. per 100-gm. rat*	*Thyroid iodine mg. %*		*Duration of experiment, days*
						$Zn(OH)_2$ Ppt.	*$Zn(OH)_2$ Supt.*	
Average	Thiouracil	.1%	3.2	1.1 μg.	33.5	0.3	1.0	10
1509–10	Thiouracil	.1%	3.3	1.4 μg.	10.7	26.6	1.4	10+14
1515–16	Thiouracil	.1%	3.1	13.0 μg.	8.4	37.8	1.5	10+14
1517–18	Thiouracil	.1%	2.8	15.7 μg.	8.4	68.4	6.5	10+14

TABLE 4

THIOURACIL-DIIODOTYROSINE ADMINISTRATION

(Six rats per group—10 days treatment; daily iodine intake from food—0.7 to 1.2 μg.)

Diet supplement	*Av. body wt. gain—gm. per day*	*Thyroid wt. mg./100-gm. rat*	*Thyroid iodine mg. %*	
			$(ZnOH)_2$ Ppt.	*$Zn(OH)_2$ Supt.*
Thiouracil .1%	3.1	32.6	0.8	0.4
Thiouracil .1% + diiodotyrosine .1%	3.0	29.5	0.0	78.2
	3.1	22.6	5.2	51.6
	3.4	20.7	3.6	45.0
	2.4	19.2	1.6	49.0
Diiodotyrosine .1%	3.0	8.6	98.4	14.6
	2.2	8.9	109.0	10.2
	3.4	6.6	71.8	14.5
Controls	3.2	6.7	42.6	1.4

TABLE 5

THIOURACIL-DIIODOTYROSINE ADMINISTRATION

(Six rats per group—24 days total treatment;
av. daily iodine intake from food—1.0 to 1.2 μg.)

.1% Thiouracil for 10 days, followed by 14 days' treatment with	*Av. body wt. gain—gm.*	*Thyroid wt. mg./100-gm. rat*	*Thyroid iodine mg. %*	
			$Zn(OH)_2$ Ppt.	*$Zn(OH)_2$ Supt.*
.1% Thiouracil—10 days	31	32.6	0.4	1.0
.1% Thiouracil	56	51.9	0.1	0.0
.1% Thiouracil + .1% diiodotyrosine	55	36.5	0.4	77.8
.1% Diiodotyrosine	68	19.5	28.1	2.9
Controls (24 days)	85	6.5	76.9	2.2

tyrosine survives in tissues as such or whether results are due to liberated iodide.

In a final series of experiments, thiouracil was administered to rats for 24 days, during the last 14 of which they received thyroxine in varying doses. Observations were made on thyroid weight and $Zn(OH)_2$-precipitable and non-precipitable iodine values of the glands.

Dempsey and Astwood (1943) and Reineke, Mixner, and Turner (1945) have shown that thyroxine in adequate amounts given concurrently with thiouracil will prevent goiter, loss of thyroid iodine, and lowering of metabolic rate. Our own data, in substantial agreement with those of Astwood and Bissell, indicate that three micrograms of *dl*-thyroxine, given subcutaneously once daily, will prevent any thyroid enlargement in thiouracil-treated rats over a period of 10 days, whereas to maintain a normal thyroid iodine concentration under the same circumstances 20–30 μg. are required. At this dose of thyroxine, thyroid weight was somewhat diminished from normal, suggestive of partial pituitary inhibition.

Objectives of the experiment were two-fold: first, to determine whether thiouracil would prevent collection and storage of administered thyroxine when given in excessively large doses, and, second, to determine what dose of thyroxine given an animal already goitrous and continuing to receive thiouracil would produce involution of the hyperplastic gland. Rats were maintained on thiouracil, 0.1 per cent in the diet, for 24 days. Beginning on the 11th day, the animals were given thyroxine daily by subcutaneous injection in doses of 18, 40, and 80 μg. In another experiment, desiccated thyroid in a concentration of .2 per cent in the food was administered on the 11th day. Only in the latter case and in one experiment with the 80 μg. dose of thyroxine was there evidence of toxicity from these substances. At the 18 μg. dose level, one series was done with coadministered iodide, another without added iodine. In the 40 μg. thyroxine experiment, small amounts of iodide were given throughout. None was added to the diet in the case of the 80 μg. dose of thyroxine nor during administration of thyroid orally.

Results are recorded in TABLE 6. The first two experiments are controls, one showing the concentration and distribution of iodine in the thyroid and thyroid weight of rats treated for 10 days with thiouracil alone, the other with coadministered thyroxine, 18 micrograms daily. Experiment 1422 shows the results following administration of thiouracil for 24 days. When thyroxine is injected beginning on the eleventh day of thiouracil treatment and continued until the 24th day, it may be seen that at none of the dose levels of thyroxine nor when the rats were receiving thyroid orally was there any accumulation of iodine in the thyroid except for a small increase in concentration with 80 μg. thyroxine and with 45 μg. *l*-thyroxine equivalent from oral thyroid. Failure to absorb or utilize iodine may be due either to the presence of thiouracil or to an inhibited state of the pituitary from excessive concentrations of circulating thyroxine. It has been suggested that thyrotropic hormone is as essential for thyroid synthesis as it is for discharge of hormone from the gland. It does not follow, however, that the inability of the thyroid to absorb thyroxine itself is due to the presence of thiouracil, since there is no evidence that this substance is absorbed by the gland under conditions when thiouracil is absent. These observations are in conformity with those of Astwood and Bissell, who employed the smaller doses of thyroxine in similar experiments.

To examine further the quantitative aspect of inhibitory effects of thyroxine on pituitary thyrotropic activity, two experiments were done. Thiouracil was administered for 10 days, then discontinued and followed immediately by 14 days' treatment with thyroxine alone. At a daily dose of 18 μg., iodine accumulated in the thyroid in a concentration comparable to that observed in control rats receiving no thyroxine. There is, in fact, not only no evidence of pituitary inhibition but an apparent acceleration of rate of iodine storage at this dose of thyroxine, especially when the two rat groups are compared at similar intakes of iodide.

Table 6

Distribution of Iodine in Thyroid and Thyroid Weights of Rats Given a Low and Moderate Iodine Diet Containing Thiouracil for 10 Days, Followed by a 14-Day Period during which Thiouracil was Administered Simultaneously with Thyroxine or during which Thiouracil was Discontinued and Thyroxine Given Alone. Six Rats per Group. Thyroid Weight and Iodine Concentration Estimated from Pooled Thyroids of Each Group

Exp. No.	*Diet supplement*	*Body wt. gain gm./day*	*Av. daily iodine intake* per 100-gm. rat*	*Thyroid wt. mg./100-gm. rat*	*Thyroid iodine mg. %*	
					$Zn(OH)_2$ Ppt.	*$Zn(OH)_2$ Supt.*
Average	Thiouracil .1%—10 days	3.2	1.1 μg.	33.5	0.3	1.0
Average	Thiouracil .1% + thyroxine 18 μg. daily—10 days	2.8	10.3 μg.	6.0	60.0	3.7
1422	Thiouracil .1%—24 days	2.3	1.1 μg.	51.9	0.0	0.1
1750–51	Thiouracil .1%—24 days	2.7	1.1 μg.	50.7	0.6	0.6
1754–55	Thyroxine 18 μg.—last 14 days	2.6	10.3 μg.	67.0	1.2	1.1
1798–99	Thiouracil .1%—24 days Thyroxine 40 μg.—last 14 days	2.7	9.9 μg.	29.4	0.9	1.2
1868–69	Thiouracil .1%—24 days	2.5	1.0 μg.	13.5	2.4	1.1
1872–73	Thyroxine 80 μg.—last 14 days	1.7	1.2 μg.	9.6	3.5	1.3
1849–50	Thiouracil .1%—24 days Thyroid .2% (45 μg. thyx.)—last 14 days	1.2	1.2 μg.	11.0	3.7	2.1
1509–10	Thiouracil .1%—10 days Autopsy 14 days later	3.3	10.7 μg.	10.7	26.6	1.4
1752–53	Thiouracil .1%—10 days	2.8	1.1 μg.	11.6	33.1	2.8
1756–57	Thyroxine 18 μg.—14 days	2.9	11.1 μg.	11.5	41.7	2.9
1800–01	Thiouracil .1%—10 days	2.1	9.3 μg.	20.5	6.1	1.1
1870–71	Thyroxine 40 μg.—14 days	2.0	1.3 μg.	9.6	1.8	0.9
1851–52	Thiouracil .1%—10 days Thyroid .2% (50 μg. thyx.)—last 14 days	1.7	1.2 μg.	12.4	26.3	1.4

* Does not include iodine from injected thyroxine.

In contrast, when thyroxine is administered in doses of 40 μg. daily following thiouracil treatment, little or no iodine accumulates in the thyroid. This result may be interpreted as indicating pituitary inhibition and failure of thyrotropin formation, whereas the 18 μg. dose was insufficient to produce this effect. In this connection, it may be recalled that Astwood showed that rat thyroids depleted of iodine by thiouracil did not accumulate iodine in appreciable amounts after hypophysectomy.

In a single experiment conducted with oral thyroid instead of thyroxine, no interference with iodine absorption or synthesis into protein-bound form was evident. This occurred despite an estimated absorption of the equivalent of 50 μg. *l*-thyroxine daily.

These observations may be summarized as follows: Administration of large doses of iodide to rats treated chronically with thiouracil, propyl thiouracil, and other antithyroid compounds having similar mode of action results in absorption of iodine by the thyroid, proportional over a wide range to the amount of iodide ingested daily.

Iodine so absorbed is not synthesized into protein-bound form but remains in $Zn(OH)_2$ supernatant fractions made from homogenized thyroid tissue. The form in which it exists under such circumstances is not known. Salter's (1945) suggestion that a loose combination exists between iodide and protein colloid, probably adsorptive in character, is logical. Astwood (1944–45) suggests that such a mechanism of iodide absorption and accumulation exists independently of the iodination system in the gland and that only the latter is inhibited by thiouracil.

The goitrogenic action of thiouracil and other antithyroid compounds is appreciably reduced by high iodine intakes. This action is due, we believe, not to any toxic effect of iodide but to diminished sensitivity of the thyroid cells to thyrotropic stimulation. Recovery from the goitrogenic action of thiouracil and restoration of protein-bound iodine are accelerated by high iodine intakes. These latter observations are in conformity with recent results reported by Taurog and Chaikoff (1946), showing that high iodine intakes led to increased concentrations of thyroxine iodine in rat thyroids. In agreement with the observations of Astwood, Salter, Chaikoff, and others, we find that the goitrogenic and antithyroid action of KSCN is readily inhibited by iodide even at relatively low intakes.

Diiodotyrosine in high doses is partially inhibitory to the goitrogenic action of thiouracil and, like iodide, accumulates in the non-protein-bound fraction of the thyroid when administered with thiouracil. Having no experimental evidence one way or the other at this time, we would favor Salter's view that these results are due to iodide formed from decomposition of diiodotyrosine rather than to diiodotyrosine itself.

dl-Thyroxine in daily subcutaneous injections of 3–4 micrograms given concurrently with thiouracil will prevent thyroid hypertrophy over a test period of 10 days. However, with this dose of thyroxine, iodine concentration of the thyroid diminishes, indicating either that pituitary thyrotropic action resulting from thiouracil treatment is inhibited only in part, or that hormonal iodine leaves the gland despite continuous addition to the body of amounts of hormone believed adequate for metabolic requirements. A daily dose of 20–30 μg. thyroxine administered concurrently with thiouracil will not only inhibit goiter but prevent loss of thyroid iodine as well.

Administration of *dl*-thyroxine to rats whose thyroids have been depleted of iodine by thiouracil and who continue to receive thiouracil is followed by involution of the hyperplastic gland with doses of 40 and 80 μg. daily but not with 18 μg. Oral thyroid was effective also in doses equivalent to 45 μg. *l*-thyroxine. In no case, however, was there any restoration of iodine to the thyroid either in non-protein-bound form from dietary iodine or in organic form from administered thyroxine. Failure of iodine storage by the thyroid may be due in part to the blocking action of thiouracil at the thyroid, and in part to thyroxine-induced inhibition of thyrotropin secretion at the pituitary. The latter reasoning is borne out by the observation that recovery from thiouracil is inhibited by 40 μg. thyroxine daily, whereas the smaller dose of 18 μg. appears to be without effect on the iodine absorption and iodine synthesis mechanism.

With reference to the blocking action of thiouracil on iodine absorption by the thyroid, emphasis should be placed on the conditions under which this takes place. Experimental work already cited indicates that iodine enters the thyroid gland freely when intake is high and when concentration in the medium bathing the thyroid is above certain critical levels. As has been pointed out, iodine absorbed under such circumstances remains in a non-protein-bound form as long as thiouracil or thiouracil-like conditions prevail.

If, however, iodine concentration in the environment is below certain critical levels, thiouracil prevents absorption by the thyroid. Observations of Rawson, Tannheimer, and Peacock (1944) and Franklin, Lerner, and Chaikoff (1944) in rats, as well as of Larson, Keating, Peacock, and Rawson (1945) in chicks, showed that administration of thiouracil orally for several days results in a marked reduction in the iodine-collecting capacity of the thyroid from injected radioactive sodium iodide. They also demonstrated that single subcutaneous injections of 5 milligrams of thiouracil suspension completely inhibit collection of radio-iodine by the chick thyroid within one hour after injection. These investigators showed, furthermore, that the degree of inhibition to radio-iodine collection diminished with decreasing amounts of thiouracil, varying as a linear function of the dose. Recovery from iodine absorption block induced by chronic thiouracil feeding or after single injections of the drug varies between the rat and chick experiments, but is generally complete in 24 hours. Total iodine administered as carrier with radio-iodine was uniformly 2 μg., an amount which, while it undoubtedly raised blood concentration, was insufficient to cause iodine absorption by the mechanism described earlier in this communication.

We have employed this technique as a means of evaluating quantitatively antithyroid properties of other goitrogenic substances both as to their immediate effectiveness and as to their duration of action. However, we wish to report only such details of these experiments as are pertinent to the immediate subject under consideration, namely, that iodine block at the thyroid does occur and that it involves a mechanism common apparently to all of the goitrogenic substances studied. In these experiments, radio-iodine collection by the thyroid was determined in rats and chicks following single subcutaneous injections of drug suspensions in varying amounts. This research was carried out in collaboration with Dr. R. W. Rawson and will be reported in detail jointly. Radio-iodine was injected one hour, eight hours, 24 hours and, in some cases, 48 hours after administration of the compound under study. The compounds investigated included thiouracil, propyl thiouracil, benzyl thiouracil, TC–68, TC–105, 2-aminothiazole, KSCN, and methyl thiouracil. Selection was based in part on laboratory experience with these compounds, and the compounds were also chosen because they are representative of a considerable range of antithyroid potency as judged from chronic feeding tests.

Procedure. In order to differentiate between iodine-blocking activities of the compounds studied and to exaggerate differences between dosages, iodine-absorbing capacity by the thyroid of rats used in these experiments was increased by maintaining the animals on a low iodine diet for several weeks prior to injection of the goitrogen. The diet used was the one described earlier. This was slightly goitrogenic over the pretreatment period when compared with the same diet containing iodized NaCl. It was, however, quite adequate for normal growth.

Following the preliminary low iodine dietary period, rats were divided into groups of 8–11 each. Antithyroid compounds were injected subcutaneously as suspensions in 10 per cent acacia solution. At designated intervals thereafter, one cubic centimeter of a NaI solution containing two micrograms of iodine and two microcuries of I^{131} was injected intraperitoneally. Uniformly at four-hour periods following radio-iodine injection, rats of each group were killed with chloroform, thyroids were removed, weighed, and transferred to vials containing dilute NaOH for solution. Results are expressed as per cent of collection by thyroids of control rats not treated with goitrogen but given radio-iodine at the same time.

One- to two-day-old sex-linked cockerels were given one guinea pig unit thyrotropic hormone daily for four days. On the fifth day, the chicks in groups of ten were injected subcutaneously with aqueous suspensions of antithyroid compounds in varying amounts. One hour later, 2.0 microcuries I^{131} in 1.0 cc. of a solution of sodium iodide containing 2 micrograms iodine were injected subcutaneously. Chicks were killed four hours later. Results are expressed, as in the case of the rat experiments, as per cent of control uptake.

Results. Only the data on radio-iodine collection one hour following administration of goitrogen will be presented here. Figure 2 shows the results for thiouracil, TC–68, TC–105, propyl thiouracil, and benzyl thiouracil in the rat. Arbitrarily, radio-iodine collection below 12 per cent of that of control groups was considered to be an effective block, and examination of the diagram shows that the minimal dose which will induce such a block in the doses used is 0.5 mg. for thiouracil, 5.0 mg. for TC–68 and TC–105, and .06 mg. for propyl and benzyl thiouracil. The corresponding value for methyl thiouracil, not shown on the chart, was 0.3 mg., showing that its iodine-blocking potency one hour following injection was almost twice that of thiouracil, despite the fact that in chronic tests for activity it has about the same goitrogenic activity as thiouracil. The low activity of TC–68 and TC–105, shown in these experiments,

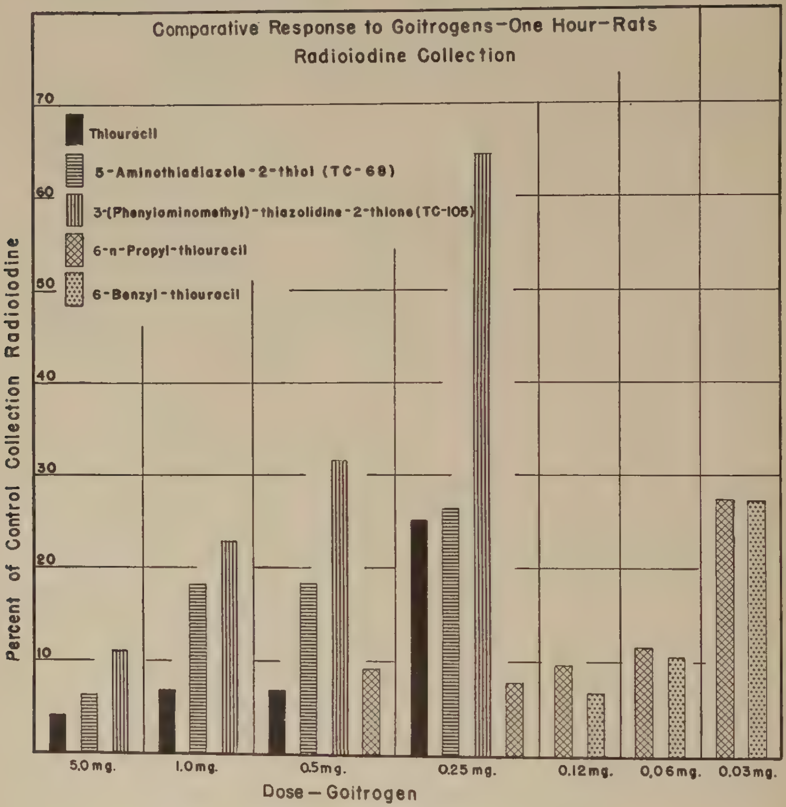

Figure 2

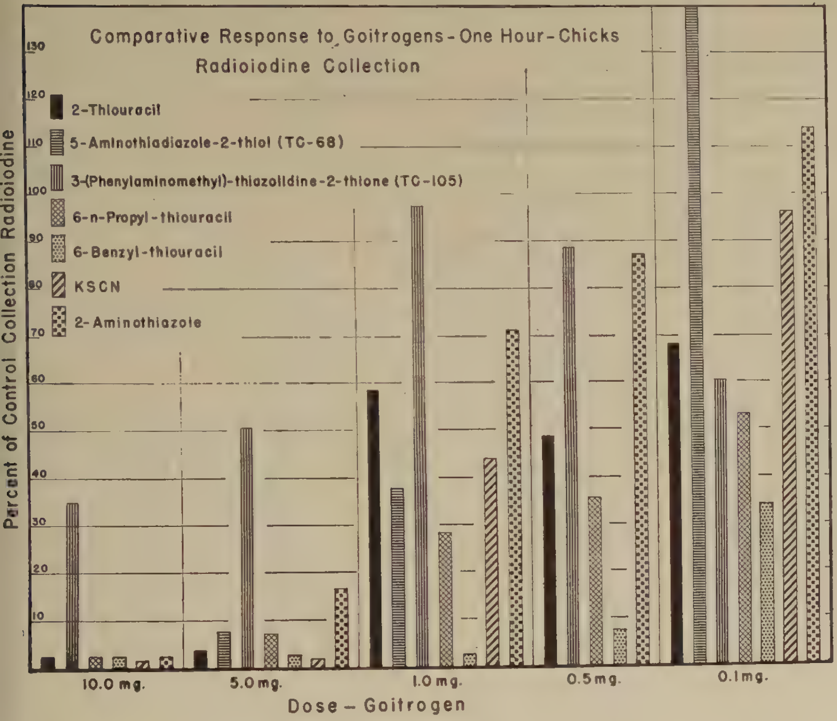

FIGURE 3

may also be contrasted with their relatively high goitrogenic activity in the chronic tests.

FIGURE 3 shows iodine collection figures for thiouracil, TC–68, TC–105, propyl and benzyl thiouracil, aminothiazole, and KSCN at various levels in chicks one hour after administration of the drug. With all compounds except TC–105, 10-mg. doses are effective in inducing an almost complete block to iodine absorption by the thyroid gland. At the 10-mg. dose, TC–105 is only weakly effective. Aminothiazole at 5 mg., while interfering considerably with iodine absorption, does not produce an effective block. This conforms with its comparatively low activity in chronic tests in rats, that is, about 15 per cent of that of thiouracil. Thiouracil, TC–68, propyl and benzyl thiouracil, and KSCN are effective at 5-mg. doses. At doses of 1.0 mg. and 0.5 mg., only benzyl thiouracil prevents absorption of injected radio-iodine in the one-hour period.

It is not the purpose here to discuss the relative potencies of these antithyroid substances as estimated by this test nor to compare the values obtained with those procured by other means. These experiments are presented only to illustrate that, when circulating iodide concentration is low, the goitrogens uniformly block uptake by the thyroid. This effect occurs within minutes after administration and persists for several hours, depending to some extent on the dose. The mechanism of block is yet to be clarified, with a possible exception in the case of KSCN, which is believed to be due either to the formation of a stable iodine-KSCN chemical combination from which iodine cannot be freed or to interference with iodine absorption by its mass effect at the cell membrane.

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Discussion of the Paper

Dr. E. D. Goldsmith (*New York University, New York, N. Y.*):

In our paper with Dr. Keston, we reported that analysis of the thyroid glands of thiourea-treated rats, 48 hours after the injection of radioactive iodine, revealed no appreciable amounts of radioactive iodine in any form, whereas thyroid glands from normal rats contained considerable quantities of radioactive iodine, chiefly in organic combination. However, we had also observed (unpublished) that, shortly after administration, large amounts of radioactive iodine could be detected in the thyroids of thiourea-treated animals. *In vitro* experiments disclosed that hyperplastic thyroids from rats receiving thiourea in their ration collected large amounts of iodide, although relatively little organically bound iodine was formed. It would thus appear that iodine uptake is not inhibited by thiourea but that it passes through the gland rapidly and does not enter into organic combination.

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MECHANISMS OF ACTION OF VARIOUS ANTITHYROID COMPOUNDS

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As long as we do not know just how thyroid function is controlled, we cannot define accurately how that function is inhibited. The production and secretion of thyroid hormone in the normal individual is nicely regulated by mechanisms, the nature of which we are only partially aware. It is possible, however, that a fuller understanding of normal thyroid function will result from a study of disturbances of thyroid activity and from an analysis of the possible mechanism of action of agencies which interfere with the normal process. The synthesis of thyroid hormone may be regarded as the primary function of the thyroid gland. Subsidiary to this are the storage of hormone within the gland and its release into the blood stream in appropriate quantities. These functions are dependent upon the complex cellular activities of the thyroid parenchyma, which, in turn, are regulated by the other influences, including the thyrotropic activity of the anterior pituitary.

An attempt will be made, in this review, to analyze these various aspects of thyroid function so as to define in what ways they may be interfered with. Substances which in one way or another depress the function of the thyroid gland may be classified under four headings: (*1*) Thyroid hormone; (*2*) Iodine; (*3*) Thiocyanate ion; (*4*) Antithyroid substances proper, compounds which interfere with thyroid hormone synthesis.

It is apparent that the structure and function of the thyroid gland are under delicate regulatory influences which insure that the quantity of hormone secreted is appropriate for the requirements of the body. We assume that, when thyroid hormone requirement is increased or when any influence tends to decrease the efficiency of thyroid hormone synthesis, there is a compensatory overproduction of thyrotropin from the pituitary, and the thyroid gland undergoes changes which we interpret as those of overactivity. Contrariwise, if the thyroid hormone requirement is reduced, or if extra thyroid hormone is artificially administered, there is a compensatory atrophy of the thyroid gland. This simple regulatory mechanism has been thought by many investigators to be inadequate to explain all of the phenomena which have been experimentally revealed. A number of investigators have suggested that there are stimulatory or inhibitory influences which act directly upon the thyroid gland and independently of the hypophysis. These direct influences include a direct stimulation of the thyroid gland by sympathetic nerve impulses; a direct inhibition of the thyroid gland by thyroid hormone or by iodine; a direct stimulus by low environmental temperature; etc. Other possible regulatory influences outside of the simple scheme outlined above include the postulate of two hormones from the thyroid gland, only one of which suppresses thyrotropin production; the theory that there is an inhibitory hormone secreted by the pituitary gland; the claim that the thyroid may inactivate its own hormone; and the theory that, under certain circumstances, the thyroid gland inactivates thyrotropic hormone, or that this hormone is inactivated by iodine and potentiated by certain antithyroid substances. Some of these possible regulatory mechanisms will have to be considered in examining the mechanism of action of substances which inhibit thyroid function. If thyroid activity were regulated solely by the simple thyrotropin-thyroid hormone interplay, one would expect that, in the hypophysectomized animal, the thyroid gland could not be influenced by any means other than the administration of thyrotropin. One would also expect that thyroid hormone itself would have no action in either stimulating or depressing the various activities of the thyroid gland in the absence of the hypophysis. Without the hypophysis, also, there should be no structural change in the thyroid gland induced by changes in iodine intake, environmental temperature, nervous influences or antithyroid agents. Experiments have been reported, however, to show that in each of these instances positive effects could be obtained under certain circumstances in the absence of the hypophysis. When referring to thyroid activity, we must be careful to distinguish between the function of hormone synthesis and the activity as judged by histological criteria. A gland may appear cytologically to be in a resting or relatively inactive state and may yet be making a normal quantity of thyroid hormone. On the other hand, hormone synthesis may be completely blocked by an antithyroid agent while the gland appears to be very active indeed upon microscopic examination.

Thyroid Hormone

The possibility that thyroid hormone may affect the thyroid gland directly has appealed to several investigators. There has been no substantiation of the claim (Pall, 1933) that thyroid hormone directly stimulates the oxygen consumption of thyroid tissue as it does that of certain other tissues; indeed, it has been claimed that there is an inhibitory action upon the respiration of thyroid tissue slices *in vitro* (Galli-Manini, 1941). Of course, the administration of thyroid to the intact animal results in regressive changes in the thyroid gland. This effect is identical with that induced by hypophysectomy, and it is tempting to conclude that the effect of thyroid is solely that of suppressing the thyrotropic activity of the hypophysis. However, the repeated demonstration, since Aron's (1930), that the concurrent administration of thyroid hormone with thyrotropin diminishes the thyrotropic effect of the latter is more difficult to explain. One could imagine that, in the intact animal, vigorous thyroid stimulation could exhaust the iodine supplied in the diet and render the animal iodine-deficient. The animal's own pituitary might then be stimulated through a lowered production of thyroid hormone from the already activated thyroid. Under such circumstances, either iodine or thyroid hormone would appear to be inhibitory to the injected thyrotropin. Further evidence for a direct effect of thyroxine in inhibiting the action of thyrotropin upon the thyroid gland is provided by the work of Van Eck (1939), and of Cortell and Rawson (1944), who have shown that, in hypophysectomized rats, thyroxine decreases the effect of injected thyrotropin.

Iodine

The several actions of iodine upon the structure and function of the thyroid gland are probably most complex, and the extensive studies of these iodine effects leave us with many unanswered questions.

It is clear that a deficiency in iodine intake and a consequent deficiency in thyroid hormone synthesis leads to the compensatory change in the thyroid gland which we recognize as hyperplastic goiter. The administration of iodine under such circumstances corrects the deficiency, permits thyroid hormone to be formed, and allows the thyroid gland to assume a more normal structure. In a sense, the administration of iodine under these circumstances evokes an antithyroid effect, that is to say, a marked degree of thyroid atrophy is induced, but of course thyroid hormone synthesis is promoted.

Two other actions of iodine upon the thyroid gland seem to be the direct antitheses of one another. The administration of iodine to persons suffering from hyperthyroidism results in a regressive change in the thyroid gland and in a diminished output of thyroid hormone. The exactly opposite situation obtains as regards thyroid hormone production when iodine is given to experimental animals or human beings with hyperplastic, non-toxic goiters. Marine and Lenhart (1909) showed that the administration of iodine to goitrous dogs had an action similar to the administration of thyroid hormone, *viz.*, the animals exhibited increased appetite, diarrhea, weakness, and weight loss for the first week or two but then usually recovered. Webster and Chesney (1928), on the other hand, found that iodine administered to goitrous rabbits induced severe hyperthyroidism which nearly always progressed to the death of the animal. These two variants of induced hyperthyroidism are also to be observed when iodine is given to human beings in goitrous districts. It would appear that the human counterpart more nearly resembles the condition seen in rabbits than that observed in dogs; that is, a self-perpetuating hyperthyroidism may be induced by the administration of iodine.

The striking effect of iodine in suppressing the thyroid and its function in hyperthyroidism remains completely unexplained. Marine and Lenhart (1911) likened this effect to that seen when hyperplastic endemic goiters were treated with iodine. They supposed that the iodine permitted the thyroid to make its normal hormone instead of a perverted one. Various mechanical theories have been proposed around the general idea that the thyroid becomes packed full of hormone when presented with such large amounts of building material and that the pressure of the accumulated secretion then shuts off the blood supply and prevents the leaching out of hormone into the rest of the body. More appealing are the theories which attempt to define more precisely just how this phenomenon is brought about. Loeser and Thompson (1934) found that, when iodine was administered to the normal animal, the quantity of thyrotropin hormone in the pituitary was reduced. This would be in keeping with the explanation that iodine shuts off the supply of thyrotropic hormone and such an effect, of course, would explain the atrophic change in the thyroid and the decreased production of thyroid hormone. However, this effect of iodine might be mediated by the thyroid hormone formed from it and not by an action of the iodine itself upon the pituitary.

The theory of a direct inhibitory action of iodine upon the thyroid gland also has substantial documentary support. The earlier experiments showing that iodine would prevent the compensatory hyperplasia which followed partial thyroid ablation were qualified by the work of Marine and Lenhart. They showed that the administration of iodine would not prevent the regrowth of thyroid tissue after partial thyroidectomy in dogs that possessed normal-appearing thyroid glands. If, however, the experiment were performed on dogs with hyperplastic, presumably iodine-deficient thyroids, then the iodine caused a reversion of the hyperplastic glands to normal ones even though large portions of the goiters had been removed surgically. They concluded that iodine would prevent hyperplasia but not compensatory regrowth of thyroid tissue. A very different concept of the action of iodine on the thyroid gland was put forth by Loeb (1920). Extensive studies by Loeb and collaborators showed that not only was compensatory hyperplasia not prevented by iodine, but iodine increased the degree of hyperplasia following partial thyroidectomy. They showed further that the administration of iodine alone to normal guinea pigs would be followed by a wave of mitotic activity associated with changes in the colloid which the authors took to indicate a stimulating action of iodine upon the thyroid gland. This effect of iodine has received little further attention and remains entirely unexplained. Later, however, Silberberg (1929) and Siebert and Thurston (1932), from Loeb's laboratory, demonstrated that the administration of potassium iodide to normal guinea pigs would inhibit partially the stimulating action of thyrotropic hormone upon the thyroid gland and, to some degree, the increased metabolism induced by thyrotropin. These results have been confirmed by Friedgood (1935), Anderson and Evans (1937), Cutting and Robson (1939), Trikojus (1939), and others. Further evidence in support of this theory was provided by Vanderlaan,

Vanderlaan, and Logan (1941), who showed that the respiratory metabolism of thyroid slices from guinea pigs treated with iodide as well as thyrotropic hormone was less than in other animals given thyrotropic hormone alone. Loeser and Thompson (1934) observed no effect of iodine in inhibiting thyrotropin in the absence of the hypophysis. Another line of evidence supporting the theory that iodine inhibits the action of thyrotropic hormone upon the thyroid gland is provided by experiments performed with antithyroid agents such as thiouracil. McGinty and Sharp (1946) have shown that, if the iodide intake is increased, there is a slight but distinct inhibition of the thyroid enlargement induced by thiouracil. This observation has been amply confirmed, and one theory which could explain the phenomenon is that the accumulated iodide either suppressed the thyroid gland directly or inhibited the action of thyrotropin upon it. There is good reason to believe that the added iodine does not act by permitting thyroid hormone to be formed. Wright and Trikojus (1946) have carried the theory one step further and have proposed that it is not the iodide which inhibits the thyrotropin, but that within the thyroid gland iodide is converted to iodine and this reacts with the thyrotropin to inactivate it. Rawson and co-workers (1946) have made extensive studies on the inactivation of thyrotropin by iodine and its reactivation by various reducing agents, including antithyroid compounds. Any theory of the mechanism of action of iodine must take into consideration the fact that added iodine in the normal animal has no detectible effect upon the thyroid gland.

Of course, one might ask: "What is a normal thyroid gland?" In order for it to be normal, there must be available an adequate iodine intake, but where is one to draw the line between an inadequate and an adequate supply of iodine? The work of Levine, Remington, and von Kolnitz (1933) showed that, as the iodine in the diet is increased from deficiency levels upward, there is a gradual decrease in the resulting size of the thyroid gland and a gradual increase in the amount of iodine stored therein. At no point can one draw the line and say where deficiency stops and excessive iodine intake begins (FIGURE 1). Similarly, Taurog and Chaikoff (1946) have shown that there is a progressive increase in the total of thyroxin iodine in the thyroid and in the protein-bound iodine of the plasma of rats as the daily iodine intake is increased up to as much as 78 micrograms per day (FIGURE 2). One might argue that, if the dietary iodine is already adequate or excessive, the addition of more iodine will then have no demonstrable effect upon the normal thyroid gland. Perhaps a more satisfying theory would not hold that iodine suppresses the thyroid gland directly or inhibits the action thereon of thyrotropic hormone, but one might state the proposition that a deficiency of iodine within the thyroid cell is a stimulus to it. The most direct evidence in support of this possibility is that of Chapman (1941), who observed an increase in the size, vascularity, and hyperplasia of the thyroids of hypophysectomized rats fed a low iodine diet.

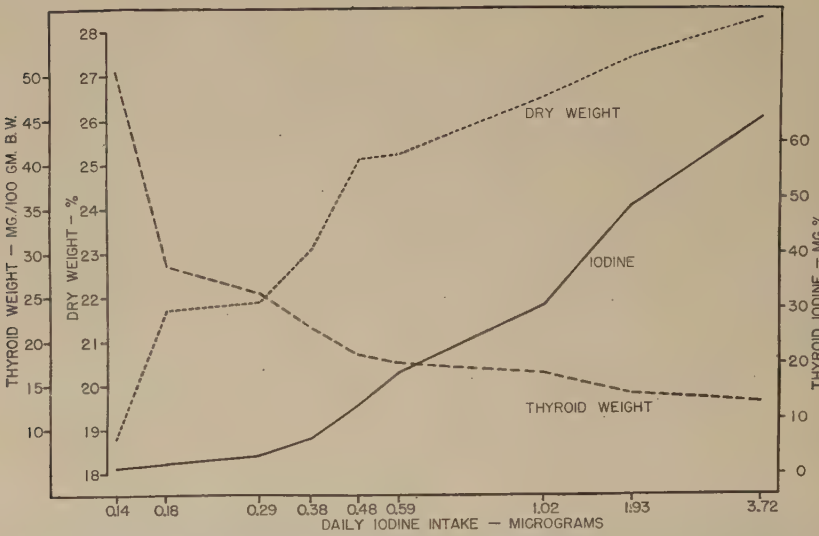

FIGURE 1. The effect of the prolonged feeding of diets of varying iodine content upon the resulting weight, dry weight, and iodine concentration of the thyroid glands of rats. The thyroid weight is expressed in milligrams per 100 grams of body weight, and the iodine concentration is expressed in milligrams per 100 grams of fresh thyroid tissue. The iodine intake in micrograms per day is plotted on a logarithmic scale. (Redrawn from the data of LEVINE, REMINGTON, & VON KOLNITZ, 1933.)

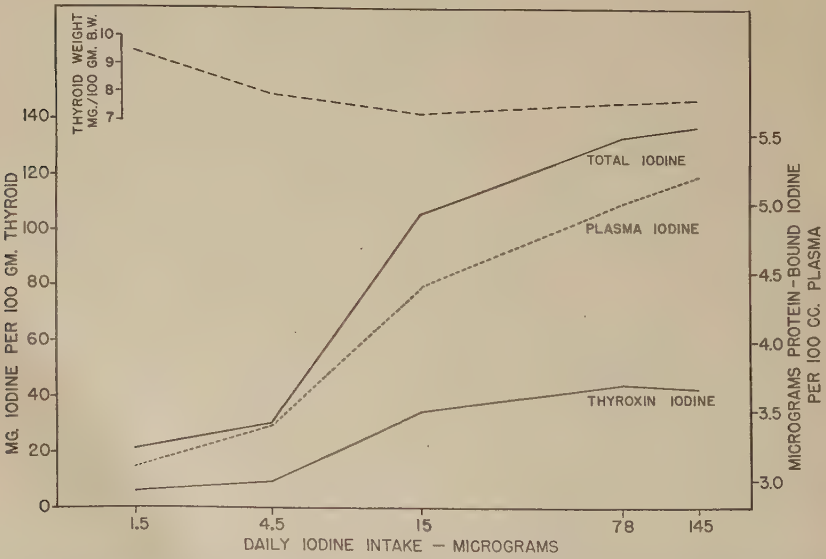

FIGURE 2. The effect of various quantities of dietary iodine upon the thyroid weight, the plasma protein-bound iodine, the total, and thyroxine iodine concentrations in the thyroid glands of rats. The thyroid weight is expressed in milligrams per 100 grams of body weight, the total and thyroxine iodine concentrations are in milligrams per 100 grams of wet thyroid, and the protein-bound iodine is in micrograms per 100 cubic centimeters of plasma. The iodine intake in micrograms per day is plotted on a logarithmic scale. (Redrawn from the data of TAUROG & CHAIKOFF, 1946.)

It would seem likely that a proper understanding of the action of iodine on the thyroid gland of experimental animals would lead to a solution of the puzzling question of how iodine benefits patients with Graves' disease. De Robertis and Nowinski (1946) have shown that, if material from the thyroid gland containing the proteolytic enzyme, which they regard as important for the mobilization and secretion of thyroglobulin, is treated with iodine, the proteolytic activity is destroyed. He proposes this as the explanation of the action of iodine in Graves' disease. It might be well to recall that the treatment of a protein enzyme with iodine is a vigorous chemical procedure, and such treatment is likely to inactivate most enzyme systems. It is doubtful that such a reaction would take place within the thyroid gland. Morton, Chaikoff, and Rosenfeld (1944) have shown that the synthesis of thyroid hormone by thyroid tissue slices *in vitro* is inhibited by large excesses of iodide ion in the medium.

The extensive work on iodine and the mechanism of its action in Graves' disease leaves us without an entirely satisfactory explanation.

Let us examine, then, the theory that a decreased quantity of iodide ion in the thyroid cell is a direct stimulus of that cell's activity. According to this theory, one should find that goiter induced by iodine deficiency should be far more striking than that induced by such antithyroid agents as thiouracil, for not only would there be compensatory hyperplasia induced by overproduction of the thyrotropin, but the gland would, according to theory, be stimulated directly by the low iodide concentration within it. The largest goiters experimentally induced are probably those of Chesney, Clawson, and Webster (1928) in rabbits fed on cabbage. If, as seems likely, the diet not only provided a goitrogen but was at the same time iodine-deficient, the findings would be in keeping with the theory. The effect of iodide on the goiter induced by antithyroid agents, such as thiouracil, is ever so much more striking in the range of partial iodine deficiency than with very large quantities of added iodine. That is, an iodine-deficient diet induces a great potentiation of goitrogenic effect, whereas iodine added to a "normal" diet only slightly inhibits the goiter. Considering, then, the effects of iodide on Graves' disease, one might suppose that in that condition an individual on a normal diet is indeed iodine-deficient. The turnover of iodine and thyroid hormone is ever so much increased, and, as shown by Curtis and Puppel (1938), hyperthyroid individuals are in negative iodine balance when eating a normal diet. Thus, one might suppose that the iodide concentration in the thyroid cell of Graves' disease is very low. This may be one link in the chain of events which leads to the self-perpetuation of hyperthyroidism in man. Iodide administration under these circumstances would restore the normal iodide concentration in the thyroid and cause a certain degree of thyroid atrophy by withdrawing the stimulus of a low iodide concentration. It must be emphasized that this is pure speculation.

Thiocyanate

Recent work demonstrates that the thiocyanate ion exerts a unique effect upon the thyroid gland, shared by no other substance yet known. The first indications of the effect of thiocyanate ion upon the thyroid gland were provided by the observations of Barker (1936) on patients with hypertension, treated with potassium thiocyanate. It was observed that, among other toxic effects, so called, a certain number of patients experienced enlargement of the thyroid gland. The patients exhibited a myxedema-like swelling of the tissues of the face, and the administration of 1 to 2 grains of thyroid daily reversed the changes within two weeks (Barker, Lindberg, and Wald, 1941). Similar observations were subsequently made by others, and in 1942 it was shown by Kobaker (1942) and by Rawson, Hertz, and Means (1942, 1943) not only that goiter may result from thiocyanate administration, but that the goiter may be associated with all the symptoms and signs of myxedema. Thiocyanate was also found to be goitrogenic in animals, but it was soon apparent that it differed from the large groups of antithyroid compounds in being readily inhibited by increasing the iodine intake (Astwood, 1943). Even the quantities of iodine contained in commercial rat food were nearly adequate to prevent the goitrogenic effect of thiocyanate given for short periods of time. Thiocyanate was also set apart from other goitrogens by the findings of Franklin, Chaikoff, and Lerner (1944), showing that thiocyanate inhibits the uptake of iodine by surviving thyroid slices *in vitro*, whereas other antithyroid agents have little effect at concentrations which effectively prevent hormone synthesis.

An interpretation of the extensive studies on iodine metabolism in the thyroid gland and of the inhibitory actions of antithyroid compounds requires that a distinction be made between *iodide* uptake by the thyroid gland and the total *iodine* accumulation. One would expect that, if there were a large amount of inorganic iodine in the thyroid gland, the administration of tracer doses of radioactive iodine would result in a very large and rapid accumulation of radioactivity in the thyroid gland. This is not the case, as the experiments of Perlman, Chaikoff, and Morton, as well as of Leblond (1942), show. Rather, there is a steady increase in radioactivity in the gland for some hours after the administration of a tracer dose (FIGURE 3). This accumulated iodide is largely protein-bound. Lein (1943) fractionated the thyroid iodine of rabbits into acetone-soluble (inorganic) and acetone-insoluble (protein-bound) iodine following the injection of 35 micrograms of iodine labeled with I^{131}. As shown in FIGURE 4, the major portion of the radioactivity accumulated slowly in the protein fraction. At the end of twelve hours, the quantity in this fraction amounted to about 1/40 of the injected dose and was still increasing. The inorganic fraction contained very little radioactivity at any time, but the maximum of about 1/200 of the injected dose was ac-

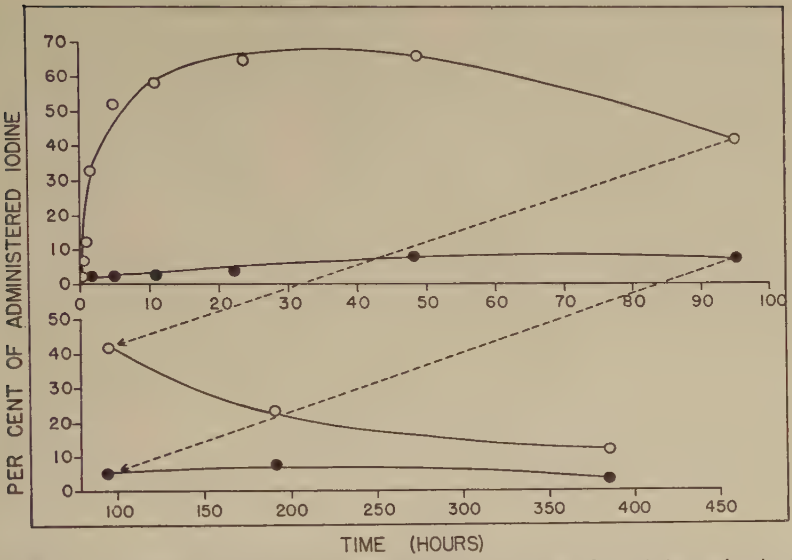

FIGURE 3. The uptake of labeled iodine by the thyroid gland of the rat. Open circles, each animal receiving a tracer dose of labeled iodine; solid dots, each animal receiving 0.03 mg. of labeled iodine. Each point represents the average of four separate analyses on as many animals. (From PERLMAN, CHAIKOFF, & MORTON, 1941.)

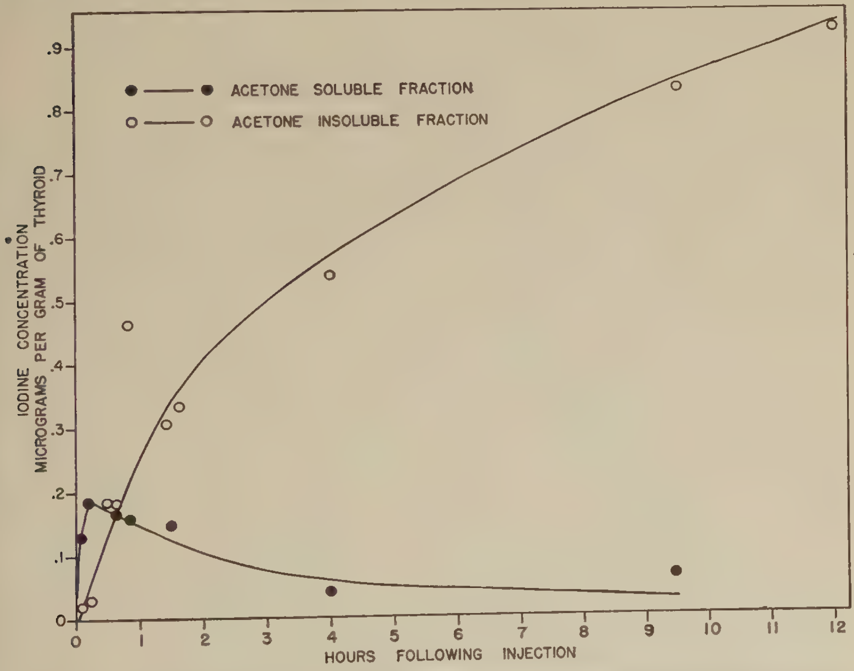

FIGURE 4. Concentration of acetone-soluble and acetone-insoluble labeled iodine in the rabbit thyroid after the intravenous administration of 35 mg. of soluble labeled iodide. (From LEIN, 1943.)

cumulated within a few minutes. The data of Morton, Perlman, Anderson, and Chaikoff (1942), and of Leblond and co-workers (Leblond and Sue, 1941; Mann, Leblond, and Warren, 1942; Leblond, Puppel, Riley, Radike, and Curtis, 1946), also indicates that the major portion of injected radioactive iodine when given in tracer doses appears relatively slowly in a protein-bound form (FIGURE 5). These experiments provide further evidence for the belief that very little inorganic iodide is held in the normal thyroid gland (Harrington, 1944). This situation contrasts sharply with that of thyroid glands depleted of their normal stores of hormone by thiouracil. If normal rats are treated with thiouracil for about ten days, the total iodine in the thyroid gland is markedly reduced, and if a dose of iodine is then given in the form of iodide, there is a very rapid uptake of iodine by the thyroid gland, reaching a maximum in fifteen to sixty minutes (Astwood, 1945; Vanderlaan and Bissell, 1946a). This markedly concentrated iodine behaves as though it were still iodide ion. Vanderlaan and Vanderlaan (1947) have shown that it is not precipitated by protein precipitants, that it readily filters through cellophane membranes, and in the ultrafiltrate both amperometric and potentiometric titrations show that it behaves in all respects like iodide ion. The magnitude of the concentration of iodide ion under these circumstances is truly remarkable. Taking an isolated case where 100 micrograms of potassium iodide is administered to 100-gram rats, assuming that this iodide is then distributed into 30 per cent of the body weight, one would have a maximal concentration of iodide in the extra

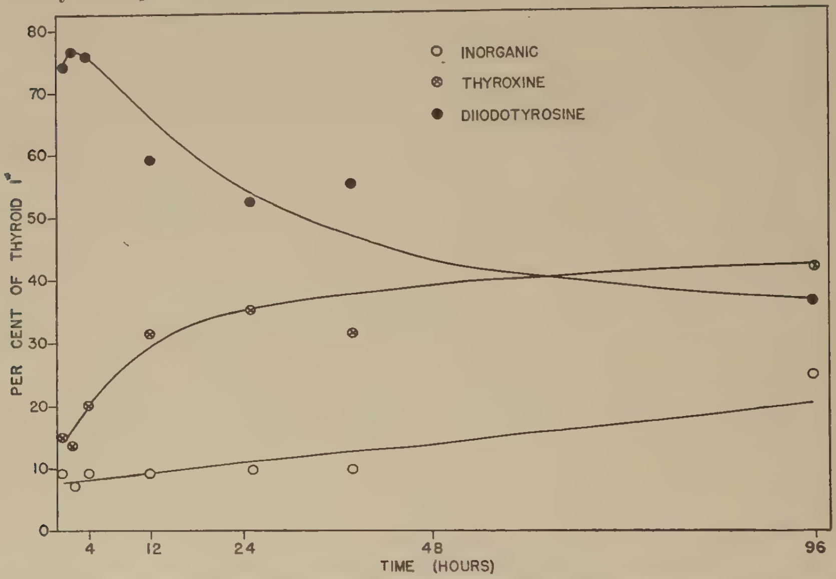

Figure 5. Distribution of thyroid radioactive iodine among inorganic, diiodotyrosine, and thyroxine fractions in normal rat. (From Morton, Perlman, Anderson, & Chaikoff, 1942.)

cellular fluid of 0.257 mg.%, whereas in the thyroid gland the concentration is 25 mg.% as a minimal figure. This represents a hundred-fold concentration. Vanderlaan and Vanderlaan have actually determined the ratio of thyroid-blood iodide after various doses of iodide, using I^{131} to label each administered dose. Following doses of 100 micrograms or less of potassium iodide, the hyperplastic thyroids of propylthiouracil-treated rats contained about 250 times as much iodide as did blood serum. The resting glands of untreated animals contained twenty-five times the amount to be found in an equal volume of blood serum (FIGURE 6). These findings show that compounds such as thiouracil do not interfere with this step of thyroid function, but they indicate that the thyroid gland, when depleted of hormone, has an enormously expanded capacity for iodide. Wolff, Chaikoff, Taurog, and Rubin (1946), as well as Vanderlaan and Bissell (1946), have shown that thiocyanate markedly inhibits the accumulation of iodide by the thyroid gland. Vanderlaan and Vanderlaan (1947) have further shown that, when as little as 1 mg. of potassium thiocyanate is administered to animals whose thyroid glands contain a large quantity of inorganic iodide, there is an immediate discharge of all of it from the thyroid gland (FIGURE 7). This phenomenon can also be demonstrated in the human being through the use of radioactive iodine (FIGURE 8). This extraordinary effect at first suggested the possibility that thiocyanate was selectively absorbed by the thyroid gland, thus displacing iodine ion. It is known that thiocyanate may be regarded as a halide, since it behaves as such in many chemical reac-

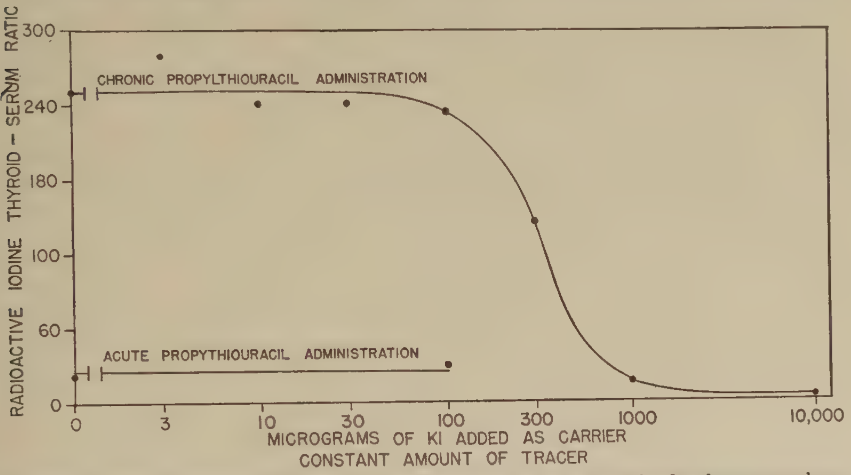

FIGURE 6. Ratio of the concentrations of radioactive iodine in the thyroid gland and serum one hour following the injection of various doses of potassium iodide labeled with radioactive iodine. The values shown in the upper curve were obtained in animals pretreated for two weeks with propylthiouracil in the diet. Those in the lower curve were obtained in previously untreated animals given a single dose of propylthiouracil before the injection of the iodine. The large, iodine-depleted glands of propylthiouracil-treated animals concentrated iodide some 250 times the serum concentration except when doses greater than 100 micrograms were given. In the normal thyroids, the iodide concentration was only about 25 times the serum concentration. (From VANDERLAAN & VANDERLAAN, 1947.)

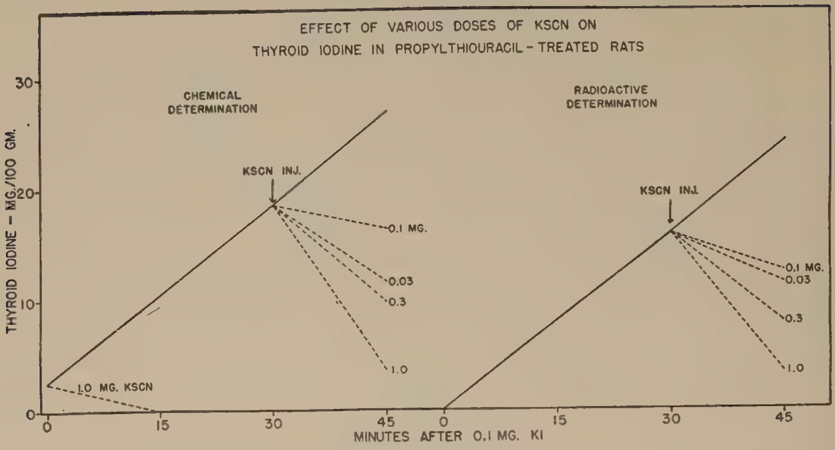

FIGURE 7. The effects of various doses of potassium thiocyanate upon the concentration of iodine in the thyroid gland of rats pretreated with propylthiouracil and injected with 100 micrograms of potassium iodide. The solid lines show the rise in thyroid iodine following the injection of iodide, and the dashed lines indicate the fall following the injection of thiocyanate. The analyses were made by chemical methods and by the use of radioactive iodine as a tracer. (From VANDERLAAN & VANDERLAAN, 1947.)

tions. It is also known that it is selectively excreted by the salivary glands, that it interferes with the excretion of hydrochloric acid in the stomach (Davenport, 1940), retards the absorption of chloride ion from the intestinal tract (Driver, 1942), and so forth. However, in no place in the body is there any appreciable concentration of thiocyanate (Corper, 1915), and it is distributed largely in the extracellular fluid. Actual measurements show that under the above circumstances there is no accumulation of thiocyanate in the thyroid gland, the blood level always remaining higher than the thyroid tissue level. The mechanism, then, whereby thiocyanate renders the thyroid incapable of concentrating iodide ion remains to be explained. The effect is so striking that it raises again the question of how important thiocyanate might be to thyroid function under normal circumstances.

The thiocyanate ion is widely distributed. It occurs in the blood and, particularly, in the saliva of normal persons and is excreted in the urine; presumably, it represents the detoxication product of cyanide. It occurs in appreciable concentrations in many plants, such as members of the *Brassica* family and Umbelliferae. Perhaps even more important than the occurrence of free thiocyanate in plants is the occurrence of substances which are turned into thiocyanate by the mammalian organism. These include the isothiocyanates, or mustard oils, the organic nitriles, and the widely distributed cyanogenetic glucosides. It seems quite possible that the consumption of foods containing these substances would render iodine-deficient a diet which otherwise would be adequate in iodine, and might explain the fact that endemic goiter exists in regions of the world where the iodine of the soil and water is abundant.

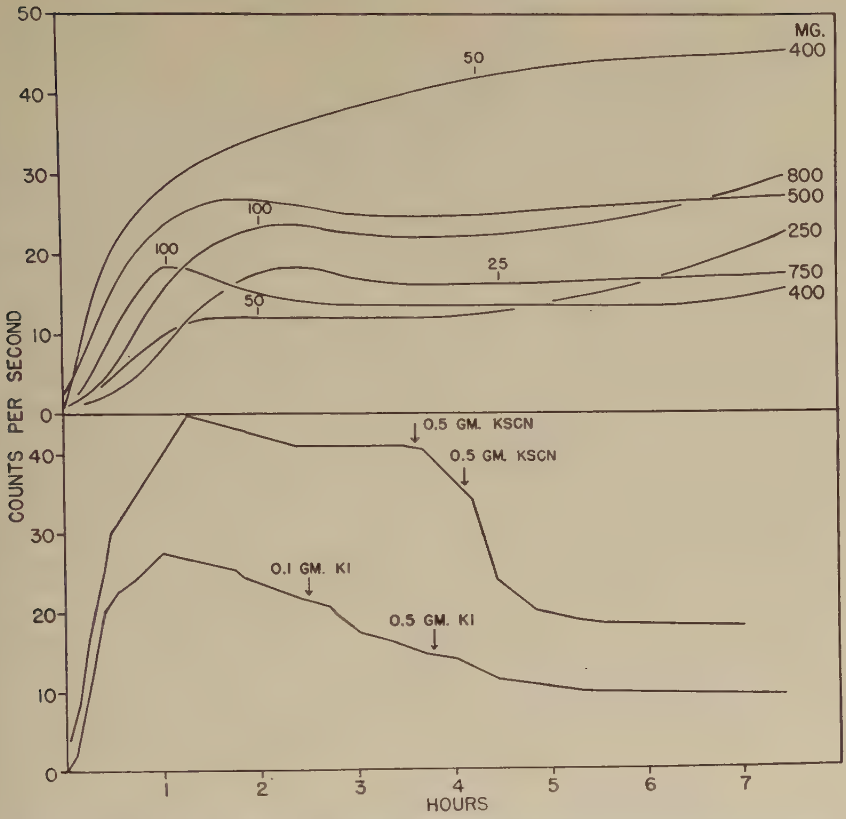

FIGURE 8. The upper part of the figure illustrates the effect of pretreatment with propylthiouracil on the accumulation of radioactive iodine in the thyroid region of normal human subjects. In each case, the first dose of propylthiouracil was given one to two hours before the 0.1 millicurie dose of radioactive iodine. The size of the last dose is shown on each curve at the time of its administration, and the total doses of propylthiouracil are given at the right side of the figure. It may be noted that four of the six subjects exhibited a fall in concentration after the maximum had been reached. The values for five of the six subjects began to rise again as the effect of propylthiouracil wore off. The curves in the lower half of the figure illustrate the effect of potassium thiocyanate and of potassium iodide on the concentrating radioactive iodine in the thyroid region in subjects pretreated with propylthiouracil. The iodine concentrated in the thyroid region presumably remains in the form of iodide which is freely diffusible. Potassium thiocyanate inhibits the iodide-entrapping mechanism, and the large doses of ordinary iodide dilute out the accumulated radioactive iodide. (From ASTWOOD & STANLEY, 1947.)

The earlier finding that thiocyanate is not goitrogenic when iodide is added to the diet might have the following explanation: The remarkable capacity of the hyperplastic thyroid gland to concentrate iodide might be of the greatest importance when the dietary iodine is low; without this special mechanism, or if it were inhibited by thiocyanate, hormone synthesis would be impeded owing to the very dilute iodide substrate. With added iodine in the diet, iodide in adequate quantities might reach the thyroid gland by simple diffusion and thus hormone synthesis could proceed at a normal rate even in the presence of thiocyanate.

Antithyroid Substances

There are a great many compounds which fall into this category. They can be divided roughly into two main groups, the most active of which are those which have in common a thiocarbonamide grouping typified by thiourea and related compounds, and secondly those with an aminobenzene grouping, the best known of which are the sulfonamides, and the most active such compounds as 4,4¹-diaminodiphenylmethane and 4,4¹-diaminobenzil. It is now generally agreed that these two groups of substances act by inhibiting the synthesis of thyroid hormone but have no action upon the iodide-accumulating mechanism and no direct effect upon the thyroid gland itself. A consideration of their mechanism of action is intimately related to the poorly understood process of thyroid hormone synthesis—the conversion of iodide into an organic form. This step presumably involves an oxidation wherein an electron is removed from iodide ion, permitting it to take the form of iodine or to be immediately incorporated into tyrosine. Now, it is abhorrent to current biochemical thought that free iodine should for any instant exist in the thyroid cell, nor can it be imagined that this free iodine could react with water to form hypoiodite and iodide or that hypoiodite could exist for any appreciable time. Nonetheless, iodide ion somehow becomes incorporated into tyrosine, and whether or not iodine or hypoiodite is ever free is a less important consideration than the fact that iodide must be oxidized. Such an oxidation could, of course, be carried out by oxygen itself, by free hydrogen peroxide, or by hydrogen peroxide catalyzed by peroxidase. The other commonly recognized enzymes of tissue presumably could not carry out this oxidation, owing to the high potential involved.

A study of the well-known reaction of thiourea-like substances with iodine has shown that, at neutral pH, free iodine is reduced by such substances as thiouracil (FIGURE 9). The rate of this reaction is so rapid that it cannot be accurately measured *in vitro*, but it is likely that it is many hundred times the rate of the reaction between iodine and tyrosine. The view put forth by Miller, Roblin, and Astwood (1945) that this reaction

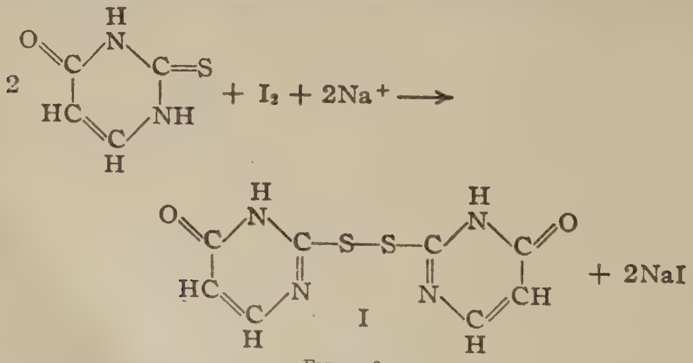

FIGURE 9.

might explain the antithyroid effect has numerous points in its favor. Each of the many thiourea-like compounds known to affect the thyroid gland reacts rapidly with iodine. There are, however, many substances such as cystine, glutathione, and thioglycollic acid, which react with iodine at a similar rate, but which do not interfere with thyroid function. It is necessary, then, to postulate that these inactive substances either do not reach the appropriate site in the thyroid cell, or else they do not reach there in a reduced form. It is interesting to speculate that the iodide-to-iodine oxidation possesses a redox potential much higher than other common oxidations in the body, and perhaps the active antithyroid agents must possess a redox potential in this range in order to be effective, having survived other common oxidative processes.

In considering the aminobenzene derivatives, it is immediately apparent that this explanation of antithyroid action cannot be applied. They react only very slowly with iodine, and could not be regarded as competitive with the iodination mechanism. Physiological experiments, however, have not yet established any clear difference between the action of the thiocarbonamide substances and the aminobenzene group, with the possible exception of the work of MacKenzie and MacKenzie (1943) and MacKenzie (1947), who showed that under certain circumstances the goitrogenic effect of sulfaguanidine is actually potentiated slightly by added iodine. Most other workers agree that increasing the iodide concentration in the diet diminishes slightly the goitrogenic effect of thiouracil and related substances. Whether this observation will lead to a distinction as to the exact mode of action between the two groups of substances cannot yet be stated.

The second step in the synthesis of thyroid hormone presumably involves another oxidative process permitting the formation of the diphenyl ether between two diiodotyrosine residues with the loss of one alanine side chain. The findings of Morton, Perlman, Anderson, and Chaikoff (1942) indicate that the hypophysis is essential to the process (FIGURE 10—compare with FIGURE 5). This oxidation is one which can also be promoted by such oxidants as iodine, hydrogen peroxide, and atmospheric oxygen, and, as Westerfeld and Lowe (1942) have shown, it can be catalyzed by peroxidase in the presence of hydrogen peroxide. It is immediately apparent that this oxidation might be very similar, if not identical, to the preceding one, and agencies which inhibit one might be supposed to inhibit the other. One could equally well reason, however, that biological oxidation can be most specific and that aminobenzene derivatives could exert their antithyroid effect by a competitive mechanism in this step due to chemical similarity between the aminobenzene radicals and diiodotyrosine (Astwood, 1943). Support for this hypothesis was thought to be provided by the fact that the administration of relatively large amounts of diiodotyrosine did not inhibit the goitrogenic effect of the sulfonamides, indicating that, though diiodotyrosine was available, this could not be converted to thyroid hormone

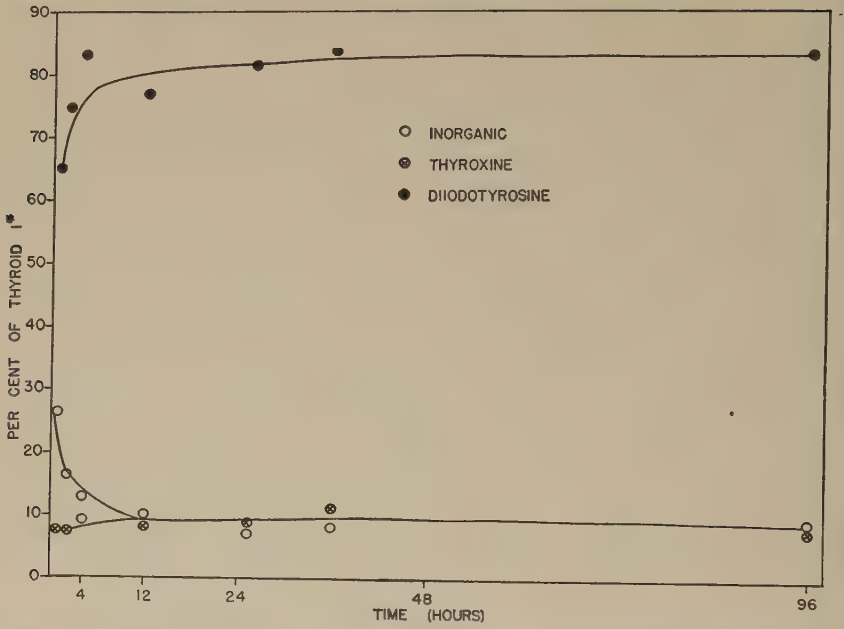

FIGURE 10. Distribution of thyroid radioactive iodine among inorganic, diiodotyrosine, and thyroxine fractions of the hypophysectomized rat. (From MORTON, PERLMAN, ANDERSON, & CHAIKOFF, 1942; compare with FIGURE 5.)

in the presence of the goitrogen. Further consideration, however, shows that this line of reasoning is fallacious, because it is highly probable that artificially administered diiodotyrosine is not utilized by the thyroid gland but is broken down in the body and only the resulting iodide ion is available to the thyroid gland (Snapper, 1938). Harrington (1944) has recently suggested that thyroxine itself is the hormone of the thyroid gland and that its synthesis goes on from the free amino acid tyrosine. When regarded from different points of view, this theory does not seem to be the correct explanation. The *in vitro* conversion of diiodotyrosine to thyroxine is a very slow process, and no matter what conditions are employed, the yields are minute (Harrington, 1934; Reineke and Turner, 1946). On the other hand, the data of Reineke and Turner (1943), showing that perfectly enormous yields of thyroxine can result from the proper management of iodinated casein, suggests that within the thyroid gland thyroid hormone synthesis goes on at the level of protein molecules rather than as amino acids. If this be the case, then artificially administered diiodotyrosine would not enter into the synthesis of thyroid hormone. We have no evidence, then, that any antithyroid substance interferes with the conversion of diiodotyrosine residues to thyroxine residues within this thyroid protein. The extensive work with radio-iodine likewise fails to reveal any inhibition at this step.

Specific enzyme inhibition is an attractive theory of the action of antithyroid compounds, and several enzymes have been suggested as important to thyroxine synthesis. The *cytochrome-cytochrome oxidase* system was shown by Schackner, Franklin, and Chaikoff (1943) to be essential to the synthesis of thyroid hormone *in vitro* by thyroid tissue slices. When this system was poisoned by azide, sulfide, or cyanide, thyroxine was not formed. However, these compounds depressed thyroid tissue respiration, whereas effective antithyroid agents did not (Lerner and Chaikoff, 1945). Morton and Chaikoff had previously presented evidence to show that intact surviving thyroid tissue was essential to thyroid hormone synthesis *in vitro*, and that homogenized tissue was ineffective. Perhaps, then, poisoning of the cytochrome system is a non-specific effect and merely serves to kill the cells the vitality of which is essential to the synthetic process. The claim that cytochrome oxidase is inhibited by thiouracil (Paschkis, Cantarow, and Tillson, 1945) has been denied by Glock (1946), who has presented evidence to show that thyroxine synthesis is not directly concerned with cytochrome oxidase. DuBois and Erway (1946), McShan, Meyer, and Johansson (1946), and Tipton and Nixon (1946) likewise found no inhibitory action of thiourea derivatives and sulfonamides on cytochrome oxidase or succinic dehyrogenase. In view of the highly specific nature of the antithyroid effect, it would be unlikely that it could be mediated by an inhibition of such a widely distributed, ubiquitously important enzyme system as cytochrome-cytochrome oxidase.

Tyrosinase is another enzyme which is inhibited by certain antithyroid compounds. These compounds include phenylthiourea, under certain circumstances para-aminobenzoic acid and sulfanilamide, and thiouracil and other thiourea derivatives (Bernheim and Bernheim, 1942; Martin, Wisanski, and Ansbacher, 1941; Paschkis, Cantarow, Hart, and Rakoff, 1944). Roberts (1945) has studied the inhibitory action of various antithyroid compounds on tyrosinase, using as substrates *l*-tyrosine, *l*-dihydroxyphenylalanine, *p*-cresol, and homocatechol. Some correlation was found between antithyroid activity and antityrosinase action. The best correlation was observed when *l*-tyrosine was the substrate. Under these conditions, thiourea, methylthiourea, guanylthiourea, para-aminobenzoic acid, and sulfanilic acid were inhibitory, while methylisothiourea sulfate and the sulfonamides were not. DuBois and Erway (1946) studied several thiourea derivatives and found that antityrosinase action paralleled closely their toxicity for rats but not their antithyroid activity. This observation and the lack of evidence for the existence of tyrosinase in the thyroid gland suggests that the antithyroid activity of these agents is not mediated by an inhibitory action on this enzyme.

Peroxidase of the several enzymes which might be concerned in thyroxine synthesis seems the most likely. It is capable of promoting the oxidation of iodide and, as pointed out by Westerfeld and Lowe (1942), it could carry out the oxidative coupling to two diiodotyrosine molecules to form thyroxine. Peroxidase is inhibited by sulfonamides (Lipman, 1941), by thiourea (Sumner and Somers, 1943), and thiouracil (Glock, 1944). Dempsey has described fine granules in the thyroid cell which give histochemical reactions typical of peroxidase and has shown that thiouracil inhibits these reactions. Glock (1944) has raised the objection that no peroxidase can be extracted from thyroid tissue. The difficulty in arriving at a decision as to whether a true peroxidase exists in the thyroid gland is caused by the numerous substances, chiefly iron porphyrin compounds, which have a peroxidase effect. Even if these substances are properly termed pseudo-peroxidases one of them could still be concerned in thyroid hormone synthesis. Dempsey's observations were confirmed by De Robertis and Grasso (1946), who found, however, that while thiourea inhibited the peroxidase reactions, sulfonamides did not. A most ingenious experiment was reported by Keston (1944). Radioactive iodide and xanthine were added to milk, and, after incubation, thyroxine containing radioactive iodine was isolated. Presumably, the xanthineoxidase of the milk acting upon the added xanthine liberated hydrogen peroxide, which in the presence of milk peroxidase oxidized the iodide to iodine, and this, in turn, iodinated the tyrosyl residues of the casein. The iodinated casein then formed thyroxine. No thyroxine was isolated if thiourea was added before incubation. This experiment may be a model of the normal mechanism of thyroxine synthesis. Randall (1946) has tested a series of 35 thioureas as well as sulfonamides and other compounds on the system H_2O_2 : horseradish peroxidase : para-aminobenzoic acid. It was found that the thiol compounds were oxidized by peroxidase and thus competed with the substrate for the enzyme and the H_2O_2. If peroxidase is normally concerned in thyroid hormone synthesis, then substances such as thiouracil could interfere in several different ways. In the reaction iodide-iodine, thiouracil could: (*1*) reduce the iodine as fast as it is formed—in other words, inhibit the enzyme; (*2*) compete for the enzyme and be oxidized instead of iodide; (*3*) inhibit the enzyme; and (*4*) compete for the available H_2O_2 so that none is available for the oxidation of iodide. It is conceivable, too, that the reaction, diiodotyrosine-thyroxine, is also mediated by peroxidase, in which case thiouracil could also inhibit by competing for the enzyme or the H_2O_2 or inhibit the enzyme by some other means.

The action of aminobenzene derivatives is also difficult to explain. We must assume that they act to prevent the organic binding of iodine, but as they do not iodinate readily and are not effective in reducing iodine they cannot act directly on iodine. Like the thioureylenes, they could inhibit peroxidase or compete with the substrate or the H_2O_2, but it is also possible that through structural similarities with tyrosine they could inhibit iodination by competition for free iodine or hypoiodite. This could be considered a competitive reaction even though very little actual iodination takes place.

Other possibilities. The well-known property of thiols, of combining with certain metals to form complexes, suggests the possibility that the synthesis of thyroxine is catalyzed by a metal or a metal-containing enzyme and that thiourea-like compounds act by containing with the metal. Reineke and Turner (1945) found that certain manganese compounds would increase the yield of thyroxine if present during the incubation of iodinated casein, and, recalling the finding that manganese increases the oxygen consumption of rats, suggested that manganese may be concerned in thyroid hormone synthesis. Considering the possibility that some unusual metal protein might be concerned with hormone synthesis, a search of the literature on tissue elements was made. No clear evidence of an unusual concentration of any element except iodine was found. Consequently, a spectrographic analysis was made of the ash of thyroid and liver of pigs. These were semiquantitative comparisons of the arc spectra made at the spectrochemical laboratory of the Massachusetts Institute of Technology. The first comparison showed a preponderance of chromium in the thyroid sample. When this was repeated with care to avoid contamination from metal instruments, no chromium was detected. As was to be expected, the liver samples contained more trace elements and larger quantities of them than the thyroid. No evidence for an unusual concentration of any element in the thyroid was found. While these findings are negative evidence that such elements as zinc, cobalt, copper, manganese, or magnesium are concerned with thyroxine synthesis, they are not against the proposition that an iron-containing enzyme is involved. The abundance of iron in tissue would obscure small differences in iron content.

Other antithyroid effects have been omitted from this review because of lack of information. Since the original description, by Moebius, of the therapeutic value of the serum of thyroidectomized animals, many agents have been claimed to have antithyroid properties. Besides various tissues, tissue extracts and plant materials, compounds such as diiodotyrosine, and bromine and fluorine derivatives, especially those of tyrosine, have been said to be beneficial in hyperthyroidism. The most recent of these was 3-fluoro-phenylacetic acid, which was introduced in Germany during the recent war. Animal experiments with this compound were recently carried out here with entirely negative results. It neither inhibited thyroid function nor interfered with the action of thyroid or thyroxine.

Woolley (1946) has recently claimed to have obtained a direct inhibition of thyroxine action by the use of various ethers of *N*-acetyldiiodotyrosine. Even with the most active compound, the paranitrophenylether, relatively very large quantities were required. The experiments would be more convincing had they been made with more specific test reactions. However, an effective thyroxine antagonist would be most useful clinically and experimentally, and perhaps Woolley's findings foreshadow such a development.

Another type of antithyroid effect is suggested by the findings of Pal and Bose (1943). They found that the thyroid hyperplasia of rats fed on a poor diet was prevented by feeding *l*-tyrosine, suggesting that thyroid hormone synthesis was interfered with through a lack of tyrosine as building material. Other workers on phenylalanine- and tyrosine-deficient diets have not commented on the condition of the thyroid gland.

Summary

In addition to various cellular and metabolic processes which it shares with other tissues, the thyroid gland has the unique capacity of accumulating iodide and using it to form thyroid hormone. All of these activities seem to be accelerated by thyrotropin from the pituitary. The administration of iodine, under certain experimental conditions, appears to depress the thyroid gland and to decrease the rate of formation of thyroid hormone. This peculiar effect is similar to that seen clinically when iodine is used in hyperthyroidism, and it remains wholly unexplained. When thyroid hormone is given, the thyroid gland is also depressed, but perhaps this is entirely a normal regulatory phenomenon mediated by the hypophysis.

Iodine metabolism within the thyroid gland, leading to the formation of thyroid hormone, can be divided into three major steps: (*1*) the concentration of iodide ion within the thyroid; (*2*) the oxidative conversion of iodide into an organic form, presumably diiodotyrosyl radicals of a protein; and (*3*) the oxidative coupling of pairs of diiodotyrosyl radicals to form thyroxyl groups.

The iodide-concentrating mechanism permits the thyroid gland to store iodide ion at a concentration several hundred times that of the circulating blood. Thiocyanate inhibits this. In the presence of thiocyanate, the gland is obliged to synthesize thyroid hormone from the iodide which passively diffuses into it from the blood. When the blood iodide is very low, because of deficient ingestion of iodine, hormone synthesis is deficient and hypothyroidism and goiter ensue.

The second major step in hormone synthesis, the oxidation of iodide, seems to be the site of action of antithyroid compounds of the thiocarbonamid and aminobenzene types. Presumably, some enzyme system is responsible for the oxidation of iodide. Of those proposed, one with the properties of a peroxidase seems the most likely. Compounds of the thiosulfur type may inhibit this enzyme directly, compete for it by serving as a substrate, compete for the available hydrogen peroxide, or reduce the iodine as fast as it is formed. More must be known about how iodide is oxidized before the mechanism of inhibition can be more precisely defined.

The coupling of diiodotyrosyl groups to form thyroxyl radicals also requires an oxidation, but just how this is brought about is unknown. Thus far, no specific inhibitor for this conversion has been discovered.

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Discussion of the Paper

Dr. C. G. Mackenzie (*Department of Biochemistry, Cornell University Medical College, New York, N. Y.*):

In connection with Dr. Astwood's reference to our early work, I would like to report that, while added dietary iodide inhibits by 50 per cent or more the thyroid enlargement produced by thiouracil on a normal diet, it does not do so with sulfanilamide, sulfadiazine, or sulfaguanidine. The response to PABA, on the other hand, is completely prevented by iodide or nearly so. In this respect, it resembles not the sulfanilamides but the thioureas. However, one can hardly assume, in the face of such different active groupings, that thiouracil and PABA inhibit the formation of thyroxin in the same way. In view of these results, it appears probable that we have at least three types of compounds, thioureas, sulfanilamides, and PABA, each of which blocks the formation of diiodotyrosine or thyroxin in a different way. This gives us three handles to grasp, three tools to employ, in attacking the series of chemical reactions involved in the synthesis of the hormone and its precursor in the body.

Second, it is of interest that, with thiouracil, one can produce almost any morphological response desired by manipulation of the levels *of iodide and the drug* in the diet. Thus, the size of the goiter can be reduced without changing the hyperplasia, or the hyperplasia may be suppressed without reducing the size of the goiter. (Endocrinology. XV: 137. 1944.)

Finally, I would like to make a plea for the use, wherever possible, of diets of known and preferably not excessive iodine content in all work on the thyroid, thyroxin, or TSH. This would greatly aid in advancing the field and permit more reliable comparison of results from different laboratories. A simple, purified diet of sugar, casein, yeast, fat, and salts could be used at least as a check on a few animals in any experiment.

Dr. Isidor Greenwald (*College of Medicine, New York University, New York, N. Y.*):

I wish to discuss two subjects mentioned by Dr. Astwood in his general summary. In the first place, no one has yet prevented the goitrogenic action of a diet of low iodine content by adding such small amounts of iodine as are found in ordinary mixed diets. As Remington and Remington[1] wrote: "The technic (of Levine, Remington, and Kolnitz) must now be considered as of a curative rather than a preventive nature."

This statement applies equally well to all other attempts to counteract the goitrogenic effect of a particular diet. In every case, the amount of iodine required to produce thyroids of the small size found in the control animals was greater than that in the control diets, and the thyroids of the experimental animals contained more iodine than did those of the controls.

I also wonder just what we are to call a "normal" thyroid. Is a human thyroid weighing 95 grams "normal," as Oswald believed? Is a rat thyroid that shows almost complete absence of iodine and colloid "normal"? Yet animals with such thyroids could "reach maturity and produce normal numbers of living young."[2]

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STUDIES ON THE METABOLISM OF THYROXINE IN THE BODY*

By C. P. LEBLOND
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IN the past, the only available methods for following the fate of both thyroid preparations and crystalline thyroxine in the body were chemical determinations or biological assay of blood and tissues. While the biological activity of tissues after thyroid administration may give a rough estimate of the amount of thyroxine present, the chemical determinations were usually iodine titrations and thus estimated not only thyroxine, but other iodized compounds (iodide, diiodotyrosine). Furthermore, for the detection of thyroxine in tissues by either biological or chemical methods, a fairly large concentration of the material is required. In order to achieve this result, the dose of either thyroid extract or thyroxine had to be so large that it probably behaved in a manner quite different from the small amounts present under physiological conditions.

Today, the availability of radioactive iodine makes possible the study not only of large doses of thyroxine, but also of minute or physiological doses such as are handled normally in the body. In the study reported here, carried out in collaboration with J. Gross, both large and physiological doses of radiothyroxine were used. The large doses were prepared by iodination of diiodothyronine with radio-iodine; the physiological, by butyl alcohol extraction of the thyroxine fractions from the thyroids of rats previously treated with radio-iodine.

The preparation of large doses of radiothyroxine was first carried out by Horeau and Süe in 1945 according to a micro-technique adapted from the classical method of Harington and Barger for the synthesis of thyroxine. The technique, which consists in having free iodine react with diiodothyronine in alkaline solution, readily yields several milligrams of radioactive thyroxine which may be intravenously injected into rats in the form of the sodium salt.

The physiological doses were prepared as follows. Rats were treated with highly active radio-iodine (0.1 to 1 microgram of iodine containing 30 to 50 microcuries of radio-iodine). Twenty-four hours later, the thyroids of the animals were removed and dissolved in 2N sodium hydroxide. After measurement of their activity on the Geiger counter, the solutions were neutralized and extracted with *n*-butyl alcohol according to Blau's method, based on Leland and Foster's finding that thyroxine is preferentially taken up by butyl alcohol. Two further washings of the butyl extract with sodium hydroxide yielded a solution containing the radioactive thyroxine fraction. After evaporation of the butyl alcohol, the

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* This work was carried out with the help of a grant from the National Research Council of Canada.

thyroxine fraction was dissolved and injected into normal and thyroidectomized rats by the intravenous route. Since the amount of thyroxine injected was extracted from the thyroids of two 100-gm. rats, it may be assumed that less than 5 micrograms of thyroxine were administered (Taurog and Chaikoff, 1946). Since, furthermore, it is known that 5 micrograms of thyroxine are used up daily by the 100-gm. rat (Dempsey and Astwood, 1943), the dose administered was considered as a physiological one.

One of the most striking features of the action of thyroxine is the ability of this substance to produce a prolonged action in the body. A single injection of a large dose of thyroxine may maintain a higher than normal basal metabolic rate for several weeks. It was, therefore, expected that this substance would be detected in the body over a long period of time. However, the classical results obtained with large doses of thyroid extract or thyroxine showed that these substances disappear from the blood quite rapidly after their administration through various routes, as demonstrated in the case of thyroid extract by Abelin and Scheinfinkel (1925), Veil and Sturm (1925), Zawadowsky and Asimoff (1927), and in the case of thyroxine by Asimoff and Estrin (1931), Müller and Fellenberg (1932). Recently, this was further confirmed by Gaebler and Strohmaier (1942), who gave 10 mg. of thyroxine intravenously to dogs. Three minutes after injection, iodine titrations showed that only half of the dose was still present in the blood. Twenty-four hours later, over 90 per cent of it had disappeared from the blood. In this laboratory, even more rapid disappearance was observed with radiothyroxine, since, after an intravenous injection of about 2 mg., only 1.5 per cent of the injected amount, on the average, was found to remain in the blood (the blood volume being taken as 4.3 cc. per 100 gm.). Boe and Elmer found, with relatively smaller doses, such as 2 milligrams of thyroxine in man, that the organic blood iodine level returned to normal within 2 hours after injection.

Even with our minute physiological dose of several micrograms of radiothyroxine injected into the rat, the whole plasma contained only about 2 per cent of the administered dose 2 hours after injection.

It is interesting to note that, while thyroxine vanished from the blood rapidly in experiments carried out *in vivo*, this substance retained all activity when added to blood and incubated *in vitro* for 48 hours (Müller and Fellenberg, 1932). It may, therefore, be concluded that the disappearance of thyroxine from the circulation is due not to a local destruction, but to a withdrawal of thyroxine from the blood, presumably under the action of some other organ.

It was recognized early that the *liver* fixes large amounts of iodine after injection of thyroid extract. Furthermore, perfusion of the liver with a fluid containing thyroid extract resulted in a marked destruction of the thyroglobulin in this extract with formation of iodide (Blum and Grützner, 1920). Later, it was more accurately proven that, with large doses of thyroxine, the liver takes up thyroxine from the blood and releases it into the bile (Kendall, 1919; Abelin and Scheinfinkel, 1925; Zawadowsky and Asimoff, 1927; Krayer, 1928; Asimoff *et al.*, 1931).

When large doses of radiothyroxine were administered (TABLE 1), the liver fixed within 2 hours as much as 10 to 40 per cent of the injected dose, with an average of nearly 20 per cent. Separation of the thyroxine fraction in this organ showed that most of the radioactivity present in the liver was still in the form of thyroxine, the balance being assumed to be iodide originating from the broken-down thyroxine. Similarly, Elmer and his collaborators carried out the chemical fractionation of the bile after thyroxine injection and observed the presence of a large amount of this substance as well as an approximately equal amount of iodide. This author concluded that half the thyroxine was broken down to iodide while passing through the hepatic cells on its way to the bile.

TABLE 1

DISTRIBUTION OF LARGE DOSES OF RADIOTHYROXINE IN THE RAT
(Percentage of the injected dose, taken by the entire organ or tissue)

	2 Hours	*24 Hours*
Plasma		
(Total estimated volume)	1.51	0.16
Internal organs		
Liver	14.00	8.10
Lung	1.45	0.34
Pancreas	0.25	0.38
Gastrointestinal tract		
Stomach wall	0.72	0.11
Stomach contents	2.85	0.15
Duodenal wall	0.44	0.40
Duodenal contents	1.13	<0.01
Jejuno-ileal wall	7.94	0.47
Jejuno-ileal contents	30.10	0.18
Colon-caecum wall	1.00	0.14
Colon-caecum contents	4.20	0.61
Faeces	0.13	68.50
Urinary tract		
Kidney	1.35	0.29
Urine	3.40	19.70
Skin	3.70	1.35
Muscle	10.80	1.23
Thyroid	0.10	0.19

Only 2 animals have so far been treated with physiological doses. The amount of thyroxine taken up by the liver was considerably reduced, being on the average 4 per cent of the injected dose (TABLE 2). In this connection, it is interesting to note that Kellaway *et al.* (1945) found that physiological doses of thyroxine have the same effect in normal and hepatectomized rats, while doses 3 times as large were more active in hepatectomized than in normal animals. This observation suggested that the liver plays a greater role in the detoxification of larger doses of thyroxine than of the normally circulating physiological doses.

TABLE 2

DISTRIBUTION OF A PHYSIOLOGICAL DOSE OF RADIOTHYROXINE IN THE RAT
(Percentage of the injected dose, two hours after injection)

Plasma	1.9
Internal organs	
Liver	4.4
Lung	0.6
Kidney	1.2
Pancreas	0.2
Gastrointestinal tract secretions	
Stomach content	8.5
Duodenal content	0.3
Jejuno-ileal content	4.0
Colon content	1.9
Endocrine glands	
Thyroid	0.5
Hypophysis	tr.
Adrenals	tr.
Muscles	7.1
Skin	5.1

In the *endocrine glands*, it has not been possible with physiological doses of radiothyroxine to confirm in the rat the claims of a specific fixation of thyroxine in the hypophysis as found in other species by Sturm and Schneeberg (1933) and by Joliot *et al.* (1944). In the thyroid gland, there is little radioactivity soon after administration of radiothyroxine, a fact which shows that this hormone does not normally enter the gland.

Krayer (1928) and Müller, and Fellenberg (1942) described large amounts of thyroxine in the skin. In this laboratory, it was found that a fair amount of radiothyroxine was present in the whole skin of the animal (TABLES 1 and 2), but the concentration was rather low.

A study of the excretion organs showed that the gastrointestinal tract had a much more important role than the kidney. The kidney, after the administration of thyroxine or thyroid extract, contained an appreciable amount of the active substance (Kendall, 1913; Asimoff and Estrin, 1931). However, the iodine in the *kidney and urine* was found to be in the form of iodide (Veil and Sturm, 1925; Zawadowsky and Asimoff, 1927), suggesting that thyroxine itself was hardly eliminated in the urine at all. It will be seen, in TABLE 1, that, 24 hours after administration, the excretion in the urine makes up close to a third of the injected dose.

Important connections were found between the thyroid hormone and the *gastrointestinal system*. This observation was rather surprising owing to the scarcity of references on the subject in the literature. It was found that, with large doses of radiothyroxine, a high proportion of the injected dose was present in all the sections of the gastrointestinal tract (TABLES 1 and 2). However, partition studies have shown that the radioactivity in the stomach contents are to a large extent in the non-thyroxine fraction, while the radioactivity was predominantly in the thyroxine fraction in the small and large intestine. Finally, following the ligation of the bile duct, pylorus, duodeno-jejunal and ileo-coecal junction, much greater amounts of radioactivity were present in the gastric and duodenal contents than in the contents of the jejuno-ileum or large intestine. It was concluded that a marked excretion of thyroxine as such takes place in the duodenum, while the radioactivity found in the stomach testifies to the excretion of breakdown products of thyroxine (iodide?).

Since, on the other hand, it is known that thyroxine deposited in a ligated intestinal loop is rapidly absorbed (Schittenhelm and Eisler, 1932), it appears likely that a large proportion of the thyroxine excreted into the duodenum is resorbed in the lower portions of the intestine. However, 24 hours after the injection of a large dose of thyroxine, a high proportion of it was found in the feces, namely, over two-thirds of the injected dose. The feces are, therefore, the main channel of excretion of thyroxine.

Summary

Thyroxine is swiftly withdrawn from the blood. This substance is distributed in the body, as indicated in TABLE 1. The liver and especially the gastrointestinal tract play an important role in thyroxine metabolism.

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Discussion of the Paper

Dr. Fredrick Gudernatsch (*New York, N. Y.*):

An item of great interest in Dr. Leblond's studies is the fact that sizable quantities of extraneous radioactive thyroxine reach the tissues as thyroxine. We may assume, from this, that the same is true for endogenous thyroxine released into the circulation from the native hormonal complex of the thyroid. At the same time, the quick discharge of radioactive thyroxine into the liver and gastrointestinal tract, after the administration of large doses, is also of interest. One wonders what the limiting factor is in the various tissues for holding adequate and safe amounts of thyroxine. In other words, how great is the "thyroxine hunger" of tissues?

Is it known whether radioactive iodine, when administered as such, has the same or a different tissue distribution coefficient as radioactive thyroxine? And does one know anything of a possible correlation between the "normal" (the average, as recorded) iodine level in various tissues and their retention of administered iodine or thyroxine? It is known that tissues contain iodine, even when the supply of I_2 to the thyroid is inadequate.

Dr. Albert J. Ritzmann (*Brooklyn, N. Y.*):

Dr. Leblond's method of using tagged thyroxine demonstrates the dispersion of the hormone systemically with findings of comparatively large amounts in some organs such as the muscle, liver, skin, heart, and gastrointestinal tract, as compared to relatively small amounts in the central nervous system and other tissues. These findings can be carried over clinically. In patients with marked hyperthyroidism, in diffuse toxic goiter, the abnormal or exaggerated findings of excess skin perspiration, irritable heart, diarrhea, altered liver function and aimless, exaggerated muscle movements link up with those organs or tissue systems in which the thyroxine content is high. Interpreted in another way, Dr. Leblond's findings show the association of high thyroxine concentration in tissues or organs where functional need and cell metabolism are greatest.

THE FORMATION OF THYROXINE IN IODINATED PROTEINS

By E. P. REINEKE

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THE iodination of proteins has been the subject of investigations covering a period of half a century. In many respects, progress in this field has gone hand in hand with the development of knowledge concerning the natural secretion of the thyroid hormone. Immediately following the discovery of iodine in organic combination in the thyroid by Baumann in 1895,[1] investigations were conducted to determine whether a thyroidal substance could be formed by the simple iodination of proteins. This early work has recently been reviewed in detail by Salter[2] and Reineke.[3] Although the early attempts were not successful in the formation of an active product, considerable information was gained on the types of combination of iodine with proteins, and the methods by which the combination could be effected. Of the various iodination methods employed, the most noteworthy is that of Blum and Vaubel,[4] who buffered their protein solutions with sodium bicarbonate in order to neutralize the hydriodic acid that forms as a side product from the substitution of iodine. With proper control of conditions, this method can now be used for the formation of iodinated proteins possessing marked thyroidal activity.

In the light of present knowledge, it is difficult to evaluate early claims of thyroidal activity in iodinated proteins, since the biological assay methods employed were not well standardized, nor were the conditions employed in forming these substances fully controlled. Blum[5] reported that his iodinated albumin produced curative effects in myxedema, but this claim was later withdrawn.[6] Following the discovery that thyroid substance will accelerate the metamorphosis of frog tadpoles,[7, 8] it was reported that comparable effects were produced with iodinated proteins.[9–11] Alkaline hydrolysis was reported to destroy the activity of such preparations.[12] Since we now know that thyroxine is relatively resistant to destruction during alkaline hydrolysis, it appears unlikely that it had actually been formed in these preparations. In fact, the effects observed were considered at the time as merely indicating a special reaction of such preparations on tadpoles, and not a true thyroidal effect.

Thyroidal Activity of Iodinated Proteins and Their Hydrolysates. With the isolation of thyroxine by Kendall[13] and its synthesis by Harington and Barger,[14] it appeared extremely unlikely that a compound of this nature could be formed simply by the iodination of proteins. Consequently, it is not surprising that subsequent results suggesting such a possibility were viewed with some skepticism.

However, Brandt, Mattis, and Nolte[15] reported that an acid-insoluble precipitate obtained from an iodinated protein after hydrolysis with barium hydroxide exerted a thyroid-like action on frog tadpoles. Likewise, Abelin and co-workers presented a series of reports[16–21] in which they gave convincing evidence that the acid-insoluble concentrates obtained from iodinated proteins after hydrolysis with alkali produced many effects that were qualitatively indistinguishable from those elicited by thyroxine.

The isolation from iodinated proteins of thyroxine in crystalline form was finally reported by Ludwig and von Mutzenbecher[22] and confirmed by Harington and Pitt Rivers.[23] The importance of conducting the iodination process under exact, but rather empirically selected conditions was emphasized. Little attention was given, however, to the possible activity of iodinated proteins prior to hydrolysis or the influence of varying the reaction conditions on the activity of the resulting product. Although it had been reported[15, 17] that iodinated proteins produced thyroidal effects only after hydrolysis, the reports of Kaer,[24] Lerman and Salter,[25] Harington and Pitt Rivers,[23] and Reineke and Turner[26] indicated that some whole iodinated proteins produce significant thyroidal effects. Consequently, our attention was turned to the possibility of increasing the activity of iodinated proteins by suitable control of the reaction conditions.

Factors Affecting the Formation of Active Substance. In the subsequent investigations, the procedure was adopted of varying single factors in the iodination and incubation processes while maintaining other conditions constant in so far as possible. Until the applicability of chemical methods for the determination of thyroxine in such preparations had been established[27] biological assay methods were employed to determine their thyroidal potency.

In making the preparations, 20 gm. of casein was placed in 700 ml. of distilled water containing sodium bicarbonate, and dissolved by stirring. The solutions were then placed in a constant-temperature water bath, and finely powdered iodine was added slowly over a period of 3 to 4 hours with vigorous stirring. The mixture was then incubated for a period of 18 to 20 hours at constant temperature, the stirring being continued throughout the process. The solutions were finally dialyzed and the iodinated protein was recovered by isoelectric precipitation.

When the sodium bicarbonate added in the above procedure was varied over a broad range in succeeding preparations,[28] good potency was observed as long as the bicarbonate used was sufficient to maintain a pH of 7.0 or above. Even though normal amounts of iodine were combined at lower pH values, there was a pronounced decline in thyroidal activity.

By a similar procedure, it was found that the amount of iodine added has a controlling influence on the amount of thyroidal substance formed. The potency of the iodinated protein increased progressively with increasing iodine input until 4.5 to 5.0 atoms of iodine had been added per mole of tyrosine in the protein.[29, 30] Iodination beyond this point resulted in pronounced decreases of activity. In the medium employed, only one-half of the reacting iodine is substituted on the tyrosine radical, the remainder being used to form hydriodic acid. Consequently, the optimal iodine input under these conditions would be slightly in excess of the amount required to substitute two atoms per mole of tyrosine in the protein. The excess would be available for oxidation in the coupling of two molecules of diiodotyrosine to form thyroxine. With a greater excess of bicarbonate, more iodine was required to reach the point of maximum potency.[31]

Proteins iodinated in a more alkaline ammoniacal medium by Muus *et al.*[31] did not reach their peak activity until considerably more iodine had been combined, and failed to show a decline in potency with excessive iodination. In the opinion of the author, this difference in results can be explained by the fact that the reactivity of diiodotyrosine as well as the oxidative action of iodine declines with increasing alkalinity of the medium.[32]

In all the earlier studies, the iodination and incubation procedures were conducted at physiological temperature on the assumption that this temperature would be optimal for thyroxine formation. Further investigation revealed that quite the reverse was true. When the temperature was increased to 60° to 70°C. during either the iodination or incubation steps and maintained at the elevated level for 18 to 20 hours,[28] a pronounced rise in the thyroidal potency of the resulting product occurred. At temperatures in excess of 90°C., little active substance was formed.

Two additional factors were found[30] to influence significantly the formation of active iodinated proteins, namely, the amount of stirring or aeration and the inclusion of any one of a series of manganese compounds as a catalyst (TABLE 1). With other conditions held constant, there is a considerable increase in the apparent thyroxine content when the amount of agitation is increased sufficiently to whip air into the solutions. Still another increase in potency occurs if the incubation is conducted in the presence of a manganese compound. Under the conditions employed, manganese tetroxide (Mn_3O_4) and the oxides obtained by the reduction of potassium permanganate with glucose exerted the greatest effect.

The combined influence of several interacting factors on thyroxine formation is shown in FIGURE 1. Manganese tetroxide appears to be effective over a considerable range of iodine concentrations. Further, the amount of iodine added remains a critical factor in the presence of manganese.

TABLE 1

EFFECT OF INCUBATION TEMPERATURE, MANGANESE COMPOUNDS, AND AMOUNT OF AGITATION ON FORMATION OF THYROXINE IN IODINATED PROTEIN

(From *J. Biol. Chem.* **161**: 613. 1945.)

Catalyst	*Stirring* *r.p.m.*	*Thyroxine content* *per cent*	*Average* *per cent*
Series I. Skim milk proteins iodinated and incubated at 37°			
None	Very gentle	0.33	
None	Very gentle	0.26	
None	Very gentle	0.27	0.29
Series II. Casein iodinated at 38–40°, incubated at 70°			
None	300	1.67	
None	600	1.73	
None	600	1.80	
None	600	1.75	
None	600	1.84	1.76
Mn_3O_4	300	1.94	
Mn_3O_4	300	1.99	1.96
Mn_3O_4	600	2.72	
Mn_3O_4	600	2.93	
Mn_3O_4	600	3.03	
Mn_3O_4	600	2.78	
Mn_3O_4	600	2.80	
Mn_3O_4	600	3.04	2.88
Oxides from reduction of $KMnO_4$	600	2.97	
	600	2.96	
	600	2.60	2.84
MnO_2	600	2.16	
MnO_2	600	2.19	2.17
Mn_2O_3	600	2.26	
Mn_2O_3	600	2.33	2.30
$MnSO_4$	600	2.00	
$MnSO_4$	600	2.13	2.07

Although it is possible to demonstrate some thyroidal activity in iodinated proteins prepared under a variety of conditions, all of the factors discussed appear to be critical and must be maintained at the optimum in order to obtain preparations of high potency.

Further information on the control of thyroxine formation is provided by results obtained in the direct synthesis of thyroxine from diiodotyrosine. It was first reported by von Mutzenbecher[33] that, when diiodotyrosine is incubated in mildly alkaline solution at 37°C. for a period of two weeks, crystalline thyroxine equivalent to about 0.1 per cent of the diiodotyrosine used initially is formed. This was fully confirmed by others[34–36] using an identical procedure. Harington[37] stated that, when diiodotyrosine was oxidized with hydrogen peroxide on the steam bath, the solution meanwhile being shaken constantly with *n*-butanol to extract the thyroxine as it was formed, a greatly improved yield was obtained.

When diiodotyrosine was dissolved in N/10 sodium hydroxide and

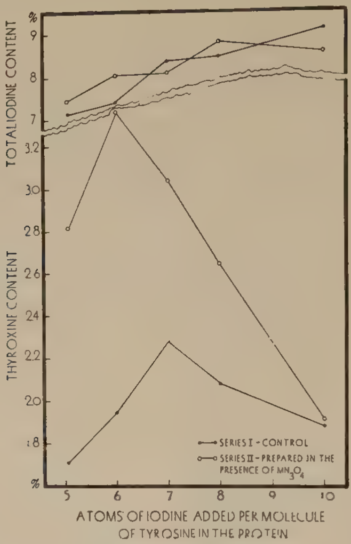

FIGURE 1. The effect of progressive iodination in the presence of excess bicarbonate and manganese oxide on the thyroxine content of iodinated casein. (From J. Biol. Chem. 161: 613. 1945.)

incubated for 18 to 20 hours with vigorous stirring or aeration,[32] the results were very similar to those obtained with iodinated casein (FIGURE 2). Quite appreciable yields of thyroxine were obtained under optimum conditions, amounting to 0.85 per cent of the diiodotyrosine incubated. If allowance was made for the unaltered diiodotyrosine that could be recovered, the net yield amounted to 2.8 per cent. The incubation temperature is highly critical, with an optimum at about 60°C. Thyroxine

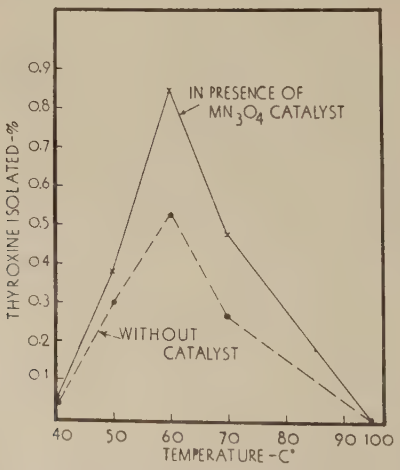

FIGURE 2. Gross yield of thyroxine isolated after incubation of diiodotyrosine at various temperatures. (From J. Biol. Chem. 162: 369. 1946.)

formation was increased somewhat in the presence of manganese tetroxide at all points in the effective temperature range. In addition, it was found that thyroxine formation was negligible unless the solutions were either stirred or aerated directly, and also that manganese tetroxide catalyzed the reaction only when air was introduced. It appears probable, therefore, that the effect of manganese is due to its acceleration of an atmospheric oxidation taking place at some point in the chemical system involved. Further investigation will be required to determine the site of action.

Nature of the Active Substance in Iodinated Proteins. From the fact that thyroxine can be isolated readily from iodinated proteins prepared under suitably controlled conditions, there can be no doubt that their thyroidal activity is due at least in part to their thyroxine content. Ludwig and von Mutzenbecher,[22] as well as Harington and Pitt Rivers,[23] reported that subsequent to hydrolysis of iodinated proteins with barium hydroxide approximately 0.1 per cent of crystalline thyroxine was recovered.

By the use of highly active iodinated casein and a similar method of hydrolysis and isolation, Reineke and Turner[38] obtained a yield of 0.424 per cent of crystalline *dl*-thyroxine. In a more recent attempt by the author (unpublished), 0.5 per cent of thyroxine was isolated. The identity of the compound is fully established by its characteristic crystalline structure (FIGURE 3), by the fact that it shows an ultraviolet absorption curve identical with that of synthetic thyroxine (FIGURE 4), by its iodine content of 64 to 65 per cent, and finally by its high metabolic potency when administered to test animals.

The maximum yield isolated actually represents about 5 times the thyroxine content of USP thyroid. However, it is only about 1/6 of the amount of thyroxine that is apparently present, as judged from the results of chemical and biological assays. The discrepancy can be accounted for, in part, in the sizable losses of thyroxine involved in its isolation and purification. In addition, there are apparently variable losses during the hydrolysis in strong boiling barium hydroxide solution that is required to liberate the thyroxine. There is also the possibility that a part of the activity is due to the presence of an as yet unidentified thyroxine-like compound.

As would be expected from its method of formation, thyroxine exists in iodinated proteins in the natural *l*-form. In the usual alkaline hydrolysis, racemization occurs, so that a *dl*-mixture is obtained. When active iodinated protein was hydrolyzed in a mixture of sulfuric acid and *n*-butanol, racemization was avoided and pure levorotatory thyroxine was isolated quite readily.[39]

The relative potency of *l*-thyroxine compared with that of the racemic mixture that is more easily available for use as a standard, is of considerable importance in evaluating the results of biological assays. Biological

Figure 3. Spectrographic absorption curves of synthetic thyroxine and thyroxine isolated from a barium hydroxide hydrolysate of iodinated casein. (From J. Biol. Chem. 149: 555. 1943.)

assays of the compounds separated by Harington from a racemic mixture indicated[40–42] that *d*-thyroxine has 1/3 or more of the activity of the *l*-form. However, it was pointed out in the original report on the resolution of these compounds[43] that the separation of isomers was probably not complete. The higher specific rotation of *l*-thyroxine tested more recently[39, 44] indicates better optical purity. Thus, it seems that the activity attributed to *d*-thyroxine could be accounted for by contamination with *l*-isomer. It should be noted, however, that Deanesly and Parkes[45] tested on *Xenopus* tadpoles a specimen of synthetic *l*-thyroxine that showed a high specific rotation, and failed to find it more active than the *dl*-mixture.

The chart shown in figure 5 is typical of results we obtained[46] when the potency of *l*-thyroxine isolated from iodinated casein was compared with that of a *dl*-mixture. In this instance, the ability of the thyroxine to prevent the increase in the thyroid weight of thiouracil-treated chicks was used as the measure of response. When the data are plotted so that the *dl*-thyroxine dosage scale is twice that used for *l*-thyroxine, the response curves are identical, demonstrating that the latter preparation has twice the activity of the former. Similar results were obtained in tests on thiouracil-treated rats, by the metabolic stimulation of guinea pigs, and by the metamorphosis-stimulating effect in *Rana pipiens* tad-

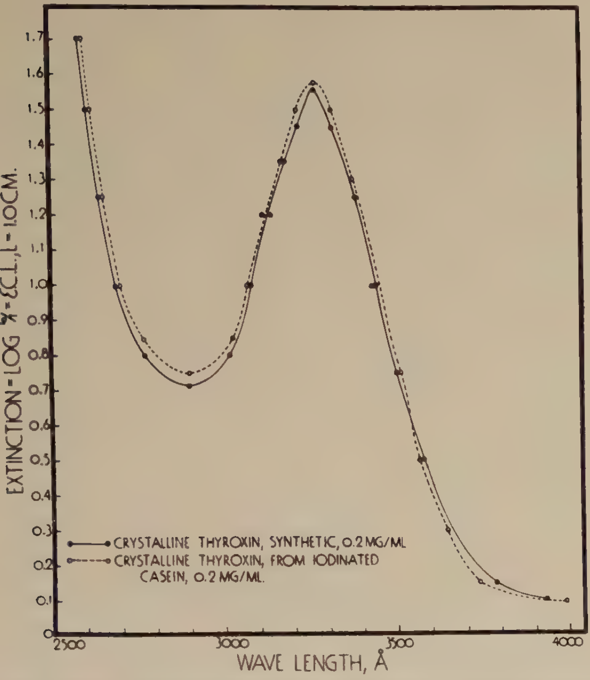

FIGURE 4. Crystalline *dl*-thyroxine isolated from thyroactive iodinated casein subsequent to hydrolysis with barium hydroxide (x 400).

poles. Thus, it was concluded, in agreement with Foster *et al.*,[44] that all of the activity of *dl*-thyroxine can be accounted for by its *l*-component. In attempting to determine the thyroxine content of iodinated proteins by biological assays, comparisons have either been made with an *l*-thyroxine standard, or, where a *dl*-standard was used, the apparent thyroxine content of the preparation under test has been divided by two to convert the value to the *l*-thyroxine basis. The biological assay of thyroidal preparations is further complicated by differences in the absorption of different substances, particularly when administered orally. Quite surprisingly, it was found[28] that, when dissolved in mildly alkaline solution, iodinated proteins are highly effective when given by injection. Therefore, in attempting to estimate the active substance actually present in the iodinated protein, it was injected and the response compared directly with that obtained with injected thyroxine.

The specificity of chemical methods for the determination of thyroxine, when applied to iodinated proteins, must be well established before the results obtained can be interpreted properly. Measurement of the acid-insoluble iodine of iodinated protein hydrolysates provides only a rough index of potency because considerable amounts of non-thyroxine iodine are included in this fraction.[45] Preliminary results with Blau's

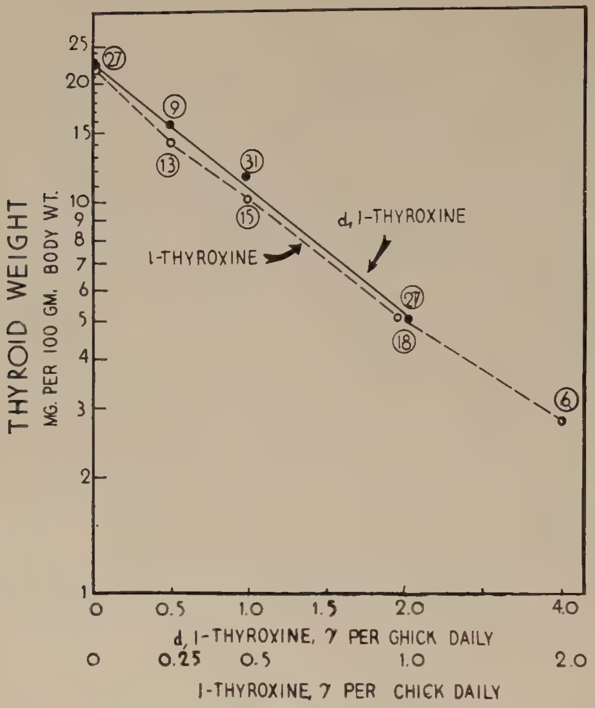

FIGURE 5. The relative potency of *l*- and *dl*-thyroxine in reducing the thyroid weight of thiouracil-treated male chicks. The encircled numerals indicate the number of animals per dosage group. (From Endocrinology 36: 200. 1945.)

n-butanol extraction procedure for the determination of thyroxine in thyroid substance failed to give good agreement with biological assays when applied to iodinated proteins. When a more vigorous hydrolysis was used, however, excellent agreement was found (TABLE 2) between the chemical thyroxine analysis and the bioassay value obtained by determining the metabolic response to intraperitoneally injected iodinated protein. The biological assay method yielded values that averaged about 8 per cent below the chemical method. This difference might be accounted for either by the inclusion of a small amount of non-thyroxine iodine in the chemical determination, or by a lower absorption, from the injection site, of the iodinated protein than of the thyroxine used as a standard. By suitable control of conditions, iodinated proteins that apparently contain about 3 per cent of thyroxine as determined by either method can now be prepared quite consistently. Biological assays made at various stages of the *n*-butanol extraction procedure indicated that all the active substance in the iodinated protein is recovered in the extraction process.

Even though close agreement is obtainable between the chemical and biological methods, the possibility of the occurrence in iodinated pro-

TABLE 2

DATA DEMONSTRATING THE CORRELATION BETWEEN THE CHEMICAL AND BIOLOGICAL ASSAY METHODS FOR THYROXINE

(From *J. Biol. Chem.* **161**: 599. 1945.)

Preparation No.	*Iodine added per mole tyrosine in protein, atoms*	*Iodinated protein injected, γ/100 g. body wt.*	*Increase in CO_2 output, per cent*	*Thyroxine found*		*Difference, per cent*
				Bioassay, per cent*	*Chemical analysis, per cent*	
1		223	25.4	2.46	2.69	−8.6
2		138	24.7	3.80	3.91	−2.8
3		176	20.8	2.46	3.06	−19.6
4		300	27.6	2.01	2.06	−2.4
5		150	20.9	2.90	3.88	−25.3
6		145	25.1	3.71	3.73	−0.5
6		161	25.0	3.31	3.73	−11.3
6		161	25.1	3.34	3.73	−10.5
7	4.51	243	21.7	1.86	2.21	−15.8
8	5.01	198	23.4	2.50	2.71	−7.7
9	5.51	201	26.8	2.90	2.69	+7.8
10	6.01	190	23.0	2.55	2.83	−9.9
11	6.51	175	23.1	2.78	3.09	−10.0
12	7.01	174	22.2	2.67	3.11	−14.1
13	8.01	191	26.4	2.98	2.83	+5.3
14	9.01	194	23.2	2.53	2.78	−9.0
15	10.01	209	25.8	2.66	2.58	+3.0
Weighted average				2.79	3.04	−8.1

* Estimated from standard response curve for intraperitoneally injected *l*-thyroxine.

teins of an active compound other than thyroxine is not wholly excluded. Such a compound, if present, however, would need to have a thyroidal activity per unit of iodine that is very similar to that of thyroxine iodine. On the other hand, if all of the substance measured by these methods is actually thyroxine, it should be possible to isolate more than the 0.5 per cent yield thus far recovered subsequent to hydrolysis.

Thyroactive iodinated proteins are effective when given orally in all species in which they have been tested to date. The oral effectiveness is considerably less than by injection and will probably vary in different species. Reineke and Turner[47] reported that iodinated protein was only about 5 per cent as effective when given to sheep orally as by subcutaneous injection. Even thyroxine given in alkaline solution was only about 12 per cent as effective by oral as by parenteral administration in this species. Deanesly and Parkes[48] point out that iodinated proteins apparently are not utilized as effectively as thyroid substance when administered orally to cattle. Species with a simple digestive system appear to utilize the active substance in iodinated proteins far more effectively than do ruminant animals. However, exact comparisons of the relative oral potency of iodinated protein, thyroid substance, and thyroxine in other species, including man, have not been reported.

Mechanism of Thyroxine Formation. When publishing their classical experiments on the constitution and synthesis of thyroxine, Harington and Barger[14] proposed the theory that thyroxine is synthesized *in vivo* by the iodination of tyrosine, followed by the oxidative coupling of two molecules of diiodotyrosine and the elimination of one side chain.

With the now well-established finding that thyroxine can be formed simply by the incubation of diiodotyrosine, or by the iodination of proteins under suitable conditions, it is proved beyond a doubt that this overall reaction will take place quite readily even without the intervention of a biological enzyme system.

However, there is little experimental evidence on the actual mechanism involved. By analogy with the findings of Pummerer *et al.*[49] on the oxidation of *p*-cresol, Johnson and Tewkesbury[35] proposed a series of reactions that would account for the oxidative conversion of thyroxine to diiodotyrosine (FIGURE 6). This would involve the oxidative coupling of two molecules of diiodotyrosine (I and II) to form the intermediate compound III. Compound III could follow one of two pathways, namely, (*a*) molecular dissociation with loss of one alanine side chain and formation of thyroxine, IV, and aminopyruvic acid, or (*b*) hydrolysis, with production of serine. Qualitative tests indicated the presence of pyruvic acid and ammonia, but not serine, as secondary products in the reaction mixture. Harington[38] developed still further the theoretical background sup-

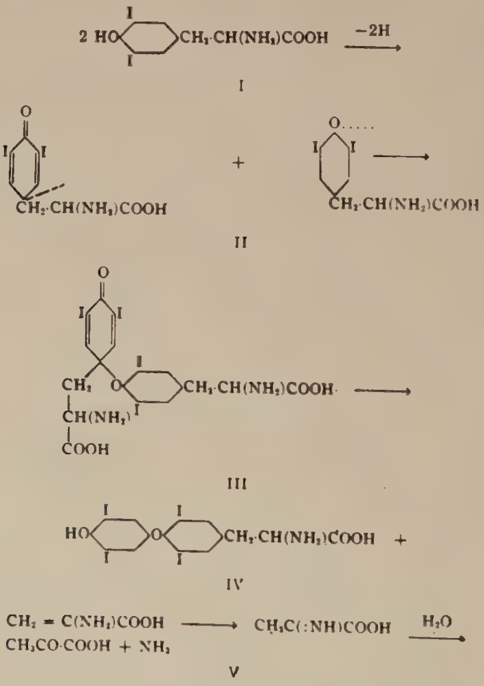

FIGURE 6. A mechanism for the conversion of diiodotyrosine to thyroxine. (From Proc. Nat. Acad. Sci. 28: 73. 1942.)

porting this theory. However, no actual evidence has been obtained on the intermediate compounds that would be necessary to fully confirm the proposed mechanism.

All the facts established thus far are consistent with the view that in the iodination of proteins, thyroxine is formed *within the protein molecule* by the oxidative coupling of two diiodotyrosine radicals, and the subsequent splitting-off of one side chain.

Although iodine can be substituted on the tyrosine combined in proteins under a variety of conditions, the secondary reactions involved in the formation of thyroxine are dependent upon the maintenance of mildly oxidative conditions in the reaction medium. It has been suggested by a number of workers that the coupling reaction involved is actually effected by the oxidative action of iodine. It is now established that thyroxine formation can be accelerated by incubation at 60° to 70°C. in the presence of vigorous stirring or aeration. The catalytic effect of manganese is apparent only in the presence of oxygen. It can easily be demonstrated that all of the iodine added combines either with the protein or as iodide during the iodination step. Consequently, no free iodine would be present for oxidative purposes during the incubation period when most of the thyroxine is formed. If we accept the idea that iodine is the effective agent in the conversion of diiodotyrosine to thyroxine, the influence of oxygen and manganese at high incubation temperatures might be explained by their oxidation of iodide to a more highly oxidized form such as hypoiodite. This compound would then be available for the oxidative coupling reaction. From the evidence now available, it seems clearly established that the thyroxine formed during iodination remains in firm combination in the protein molecule. Quite a vigorous hydrolysis with either alkali or acid is required to liberate the thyroxine so that it may be isolated in crystalline form. Furthermore, no loss of potency is observed after long-continued dialysis of the iodinated protein, indicating again that the thyroxine is combined in a large non-dialyzable molecule.

Our knowledge of protein structure is, of course, too meager to justify much speculation on the mechanism whereby two diiodotyrosine radicals, both of which are already combined in a protein molecule, could undergo the coupling reaction involved in the formation of thyroxine. It seems reasonable to believe, however, that only a certain proportion of the diiodotyrosyl radicals would be arranged spatially in such a position that they could undergo the reaction.

It can be calculated, for example, that in casein containing 5.65 per cent of tyrosine the theoretical thyroxine yield, if all of the tyrosine were iodinated and subsequently converted to thyroxine, would be about 10.6 per cent. Iodinated casein containing slightly more than 3 per cent of thyroxine-like substance, as determined by chemical analysis, can be prepared quite consistently, but no method of treatment has been found that will increase the conversion much beyond this point. The formation of additional thyroxine may, then, be impossible because of spatial incompatibilities.

Many parallels could be drawn between the formation of thyroxine in iodinated proteins and in the thyroid gland. In both instances, it appears well established that the synthesis consists, first, of the substitution of iodine on the tyrosyl radicals of the protein, and, secondly, the oxidative coupling of two diiodotyrosyl radicals within the protein molecule to form a thyroxyl radical.

Both reactions will take place in proteins iodinated artificially, without the intervention of enzymes. In the process occurring *in vivo*, it would presumably be necessary to have an enzyme system capable of oxidizing iodide to iodine to permit its substitution on tyrosine. Whether the oxidative coupling reaction involved in the formation of thyroxine from diiodotyrosine is actually effected by hypoiodite or a more highly oxidized form of iodine in either iodinated proteins or the thyroid, remains to be established by further investigation.

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Discussion of the Paper

Dr. Samuel Dvoskin (*Columbia University, New York, N. Y.*):

The experiments of Dr. Reineke have clearly elucidated the optimal conditions under which thyroxine is formed *in vitro* by the iodination of proteins. It may be of interest to present the results of recent experiments with the rat and chick, in which elemental iodine was injected subcutaneously *in vivo*.

Injections of a solution containing elemental iodine served to reinitiate bone growth and increase body length in young thyroidectomized female rats. Equivalent dosage of iodide only exerted minimal effects. In normal young female rats, the elemental iodine injections caused a marked decrease in cell height of the thyroid epithelium and a decrease in the weight of the thyroid gland. Iodide injections, in equivalent dosage, failed to alter gland weight and only slightly lowered cell height. In thiouracil-fed chicks, or thiouracil- or sulfadiazine-fed young female rats, the subcutaneous injection of a solution containing elemental iodine caused a complete inhibition of the goitrogenic effects. Iodide injections, in equivalent dosages, failed to decrease the thyroid cell height, but partially reduced the thyroid gland weight.

Oral administration of solution containing elemental iodine was no more effective than oral administration of iodide solutions in influencing thyroid weight and structure in normal or thiouracil-fed rats.

From the evidence, it appears that the subcutaneous administration of elemental iodine to the rat and chick has an action similar to thyroxine. It is possible that the action of elemental iodine results from the formation of an iodoprotein, *in vivo*, having thyroxine-like action. However, no evidence is at hand at present to prove this hypothesis.*

DR. VICTOR M. TRIKOJUS (*University of Melbourne, Melbourne, Australia*):

Since the lactic acid analogue of 3:5-diiodotyrosine has been shown by Foster and Gutman to be a metabolite of the amino acid, and Saul and Trikojus[1] have demonstrated its conversion by incubation to the corresponding analogue of thyroxine, it is pertinent to ask whether such a transformation would be possible in the body. Salter[2] has referred to the presence of a substance in hydrolysates of iodinated serum protein similar to thyroxine as regards its iodine content, but being nitrogen-free.

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DR. FREDRICK GUDERNATSCH (*New York, N. Y.*):

The new results which Dr. Reineke obtained in several species, amphibian, avian, and mammalian, when assaying his various iodinated proteins for thyroid-hormone activity, are very impressive. The formation in such proteins of the actual hormone, thyroxine, is now well understood. Very early, experimenters had realized the necessity of iodine for the physiological activity of the thyroid hormone, and especially the importance of the combination of iodine in organic form. Some investigators, however, maintained that the tadpole effect could be elicited by uncombined inorganic iodine. These views were found to be erroneous, for it was soon recognized that the animals treated with I_2 were maintained on protein foods, the latter being fed in association with iodine. Iodized peptides also elicited a semblance of the tadpole effect. Further, the possibility (almost certainty) that some inorganic iodine, after entering the tadpole, would become organically combined, could not be ruled out.

In 1916/17, we carried out numerous series of tadpole experiments, later interrupted by the War, with fractionated thyroid derivatives (nucleoproteins, globulins, coagulable proteins, etc.). These elicited the typical thyroid effect, albeit in varying degrees, nucleoproteins being the most potent ones. Unfortunately, we equalized the concentrations used according to the nitrogen, not the iodine, content of the fractions. However, the connection between iodine content and activity was readily seen. When graded according to their iodine content (mg./cc.), the fractions lined up exactly as when arranged according to hormonal potency. Yet, "it is not the iodine itself which provides this activity, but the special coupling of iodine with some particular protein fraction, the most potent combinations being those present in the thyroid. Thyroxine contains only twice as much iodine per molecule as diiodotyrosine, yet it is not just twice but several hundred times as active as the latter. As an example, tadpoles will respond to a determinable minimum quantity of thyroxine, but when we treat them with twice the quantity of diiodotyrosine they will show no thyroid response whatsoever, though getting the same amount of iodine. We would have to apply a much higher (more than a thousand times higher) concentration of diiodotyrosine. Likewise, tetra-iodothyronine (thyroxine) with its four I atoms is far more than twice as potent as diiodothyronine with two I atoms, though otherwise the molecules are similar."*

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* Dr. S. Barker has recently presented evidence that iodoproteins are formed at the injection site.

The effectiveness of iodine when coupled with protein constituents was again shown in later experiments extending over a number of years (Gudernatsch and Olive Hoffman, 1929–36), when we studied the effects of amino acids, singly and in various mixtures, in tadpole development. Some of the simpler acids proved to be an adequate food for mere maintenance, some of higher molecular weight (arginine, lysine, cystine) supported growth, while the aromatic acids (phenylalanine, hydroxyphenylalanine, tryptophane) showed signs of a differentiation effect. All are alanine derivatives, the same as diiodotyrosine and thyroxine. In some experiments, iodine was added to the solution of these acids and the animals were reared in very dilute mixtures. The rate of development became more rapid in every case. In increasing degree of effectiveness, the best groups ranged as follows: tyrosine alone; phenylalanine+iodine; tryptophane+iodine; tyrosine+iodine. The next step toward a much greater and true hormonal effectiveness would be diiodotyrosine and thyroxine. Dr. Reineke's iodinated proteins would range at the true hormone end of such a graded series.

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* From: F. GUDERNATSCH. Endocrine and Amino Acid Studies in the Physiology of Development -A Review of the author's experiments. 1936.

THE BIOCHEMISTRY OF THE THYROTROPIC HORMONE

By A. ALBERT
Endocrinology Laboratory, Section on Clinical Physiology, Mayo Clinic and Mayo Foundation, Rochester, Minnesota

THIS paper will review the present status of the biochemistry of the thyrotropic hormone and present some recently published and some unpublished results as they are pertinent to the major topics of this field. After a few introductory concepts, the following major topics will be considered: bioassay, preparation and purification, thyrotropic hormone in body fluids, and chemical reactions. Since there are more than a thousand references to the thyrotropic or thyroid-stimulating hormone (TSH), there is no intent to prepare an exhaustive review. Four excellent recent reviews have considerably lightened this task; one covers the older literature, two deal with the chemistry of the hormones of the anterior lobe in general but contain sections on TSH, and one discusses the variations of TSH potency in the pituitary of various animals.[1–4]

General Concepts

TSH may be defined as a substance obtained from pituitary tissue which, when given parenterally in proper dosage to various vertebrates, induces specific effects on the thyroid consisting of secretory alterations of the cytologic components of the follicular cells, hypertrophy and hyperplasia of the epithelium, vacuolization and resorption of colloid, loss of hormonal iodine, and increase of vascularity and of the size of the gland. As a result of such marked stimulation, there occur a number of secondary effects due to increased liberation of thyroid hormone, such as increased basal metabolism, loss of hepatic glycogen, acceleration of molting in urodeles, and acceleration of metamorphosis of amphibian larvae.[5–32] The possible extrathyroidal effects of TSH will not be discussed. The hormone is found only in the anterior of the three lobes of the pituitary and the evidence is overwhelming that a substance having identical or comparable properties is found in representatives of all vertebrate classes.[4] Whether the acidophiles or basophiles of the anterior pituitary form the thyrotropic hormone is an unsettled question, there being almost equal evidence for either cell type as its source.[33–54]

TSH is regarded as a separate pituitary hormone, distinct physiologically and chemically from all other known hormones of the pituitary gland. However, suggestions have been made that TSH is not the only hormone of the pituitary affecting the functional activity of the thyroid. Hypophyseal, thyroid-inhibiting substances have been postulated[55–60] but not confirmed.[61] Other workers have suggested two kinds of TSH, one inducing histologic stimulation of the thyroid and another increasing the weight of the gland.[62–64] The existence of still different kinds of TSH has been suggested: one, a heat-stable hormone effective in amphibian metamorphosis and another, heat-labile, effective on the mammalian thyroid.[9–11, 65] Another interesting suggestion is that, depending on the animal source, TSH may exist as different proteins, having different specific activities and probably also different molecular weights.[2, 3] Finally, the relative species specificity of TSH (that is, the diminished efficiency of TSH prepared from the pituitary of one animal when tested on the thyroid of a distantly related animal) may also be construed as indicating different types of TSH.[66–70] For the present, TSH will be regarded as a single thyroid-stimulating hormone, leaving open the question of the number of hormones and chemical dimorphism. Although the evidence is not uniform, it is generally agreed that no gland other than the pituitary manufactures TSH.[71–74]

The content of thyrotropic hormone in terms of one or another type of unit representing activity per gram dry or fresh weight of either anterior lobes or whole pituitary has been assessed for various animals. Despite the discrepancies among the various units employed, the following list, not to be regarded with undue confidence, can be arranged reading from highest to lowest concentration of pituitary TSH: frog, sole, rat, mouse, dog, pig, sheep, toad, beef, turkey, man, horse, rabbit, cat, pigeon, chick, guinea pig, hen.[4] This order is of great interest in that there seems to be a direct relationship between the concentration of thyrotropin in the pituitary and the histologic appearance of the thyroid, namely, the more active the thyroid of an animal appears histologically, the greater will be the concentration of thyrotropic hormone in the pituitary of that animal.

If this general concept is valid, certain interesting corollaries have been and can be postulated. First, the sensitivity of the thyroid to exogenous TSH should be greatest in those forms possessing the smallest concentration of pituitary TSH, and therefore such animals should be superior forms for bioassay. The day-old chick and the guinea pig, which are among the lowest on the list, have indeed been considered excellent assay animals (at least among warm-blooded animals), while the forms having active thyroids and high thyrotropic hormone concentrations (rats) are refractory and therefore not as useful for this purpose. Another corollary may be that the rapidity of effect of the goitrogenic agents may be greater in those forms having a high concentration of thyrotropic hormone than in those having a lower concentration.[75] Some forms may actually be so lacking in thyrotropic hormone that goitrogens might be completely ineffective, as perhaps in certain axolotls and in the silver strain of dwarf mice. Possibly, the ability to trap iodine may be related to the TSH content of the pituitary. Data from the comparative physiology of the thyroid would be of value to test the correctness of these concepts.

The general relation between the pituitary and the thyroid is regarded as being reciprocal. Administration of thyroxine is followed by a reduction of the TSH content of the pituitary. On the other hand, administration of TSH induces a marked depletion of the thyroid hormone content of the thyroid. These are net effects, since the concentration of a hormone in a gland at any time represents the difference between the amount of hormone produced during a given time and the amount of hormone secreted during that same interval. As far as the pituitary is concerned, it is not known whether thyroxine induces a diuresis of TSH, a diminution of TSH production and secretion, or some other result. In the case of the thyroid, however, it is definitely known that administration of TSH causes a depletion of thyroid hormone by inducing a marked outpouring of hormonal iodine from the gland. Presumably, during this short interval, there is little to no effect on production rate. Other factors, such as sex, reproductive activity, season, diet, drugs, toxins and variations in external environment, which affect the relative amounts of thyrotropin, have been reviewed elsewhere.[4]

Bioassay of TSH

Several of the current methods of assay of TSH are shown in TABLE 1.

TABLE 1

SOME METHODS OF ASSAY OF TSH

Reference	*Test animal*	*End-point*	*Arbitrary sensitivity*
12–21	Guinea pig	Histologic	1
1, 77	Guinea pig	Histologic	1
95	Guinea pig	Weight	10
89	Guinea pig	Weight	4
96–98	Guinea pig	Cell height	<1
99	Hypophysectomized rat	Metabolism	>1
89	Chick	Weight	<1
100	Chick	Iodine loss	0.1
85	Chick	Cell height	0.25
101	Tadpole	Metamorphosis	0.01
102	Guinea pig	Cytologic	0.001

When no data were given, I have estimated, however incorrectly, the relative sensitivity of the method, that is, the minimal amount of TSH necessary to produce a measurable effect. It is to be noted that the guinea pig and the chick were used more than any other animal, in accordance with the postulates made previously. In general, the plan of all these methods was to prepare serial dilutions of the substance to be tested, to inject them into animals one or more times a day for from one to six or more days, and then to determine one of the many end-points indicative of a thyrotropic effect. The least amount, or a multiple thereof, of the substance necessary to produce an effect has generally been regarded as a unit.

For various reasons, it is not possible to convert one unit accurately to another. Too many modifications of each main method have been proposed and few of the proposals have been accompanied by quantitative data showing if, and how much, the proposed change affected the size of the unit to be supplanted. There is an astonishing lack of quantitative treatment of much of the data. Finally, although a standard such as the international standard is lacking, nonetheless a standard of one's own devising has rarely been employed concurrently with assays.

A few examples of the chaotic conditions will be given. In the guinea pig assay, for example, there is still controversy as to whether animals weighing 200 to 250 gm. give as reliable results as those of 100 to 150 gm.[76–78] Special diets,[79] special forms of pretreatment with various thyroid depressants,[80] and the use of special agents like colchicine to facilitate counting of mitoses[81] have been suggested in attempts to improve the bioassay. In the chick, the number of modifications of the method originally proposed by Smelser,[82, 83] utilizing thyroid weight of the day-old cockerel, is appreciable. There are now more than a half-dozen chick units.[2, 84–92] The specificity of the various kinds of end-points is debated. Furthermore, a number of authors have stated that the thyroid weight of the chick cannot be used as a measure of TSH since large thyroids may be encountered showing no histologic evidence of stimulation and, conversely, small thyroids may be found showing intense stimulation. The variations between strains of chicks and even within the same strain have been found to be enormous.[93]

Recent experience with the chick method has shown that the aperiodic variations, the effect of temperature, and the effect of season of the year are very large and that it was impossible to establish a fixed unit.[94] Nonetheless, the general method utilizing chicks can be modified so that it is satisfactory for certain types of investigation. For example, ten to twenty white Leghorn cockerels, one day old, were injected once daily for three days in each assay. The weight, mean cell height, and iodine concentration of the thyroid glands have been used as a measure of TSH potency. The relationship between dosage and effect can be seen by reference to curves published previously.[94] The exact values for these three end-points were not necessarily obtained at another time, when presumably the same dosages of TSH were given, but the type of dose-response curves was the same. This indicates that the response to apparently the same dosage of TSH is not constant or consistent, and, consequently, the so-called chick unit is a "freely floating" variable. The use of fixed units was deliberately avoided by the simple expedient of using a standard with each assay. The results of unknowns are given in terms of percentage deviation from the standard. These percentage deviations can then be compared with other deviations similarly calculated, but the exact values for thyroid weight, mean cell height, and iodine concentration cannot be so compared. It is of interest that the weight, iodine concentration, and mean cell height methods of evaluating TSH potency were in close agreement with one another. While in borderline results a divergence may exist between weight and histologic end-points, which is partly understandable since the thresholds of these responses are different, no such divergence occurred when the dosage of TSH was sufficient to induce weights of thyroid between 4 and 9 mg.

It is not intended here to discuss the relative merits of each assay.[1, 12–21, 77, 85, 89, 96–102] From TABLE 1, it can be seen that the current methods are capable of detecting from about 0.1 to 10 arbitrary units or a range of a hundred-fold. The tadpole test and especially the cytologic test of De Robertis, although they have not yet been used widely enough, are promising since they represent methods useful for detection of the small amounts of TSH which may exist in blood and urine, and also of the high concentration of TSH in pituitary tissue.

In general, however, the current bioassay of TSH is unsatisfactory, especially if the various methods are compared with what one would regard as an ideal method. To rectify matters, an international standard of TSH (not of pituitary powder, which is available) should be established promptly. This should be a simple matter since TSH has been prepared in sufficient purity to serve as a standard. Although all difficulties may be solved by the use of this standard in conjunction with any of the current methods, there are, nevertheless, a certain number of attributes of which none of the current methods possesses all. These attributes are specificity, objectivity, sensitivity, convenience, and precision.

Specificity of action can be assured by the use of hypophysectomized animals. Objectivity can be achieved by the use of some specific action on the thyroid, which would be measured by an objective method. Histologic changes, unless they are striking, are not sufficiently objective, but a chemical determination of iodine concentration would be quite acceptable. Sensitivity can be achieved by appropriate choice of animal and end-point. The sensitivity should, if possible, be in the neighborhood of 0.001 arbitrary unit, so as to make the method widely adaptable. Convenience requires that the assay should be completed within a few hours, or at least during the same day, and not after a week of injections. Convenience also is to be considered in deciding on the other qualities of the assay. For example, the use of such an end-point as a determination of mean cell height can hardly be classified as a convenient method.

Although a few authors have treated their data[89, 103] by the usual mathematical methods applied to bioassay procedures, precision is the one factor which most methods thus far lack. The end-point to be used having been determined, it is a relatively simple matter to determine its variance in control animals. Having established a dose-effect relationship, one can determine how much of a difference in effect is safely above the normal variation of the controls. The minimal amount of hormone producing this effect is the minimal detectable dose. It is helpful to have a dose-effect range, however limited, in which the effect or a transformation of the actual response, such as the probit or logit method, is a linear or logarithmic function of dose. It is then possible to determine how small a difference in dose can be detected by the method. It is also possible, then, to interpolate any unknown value in terms of the concurrently administered standard without having to waste time and animals balancing the known *versus* the unknown. The use of serial dilution, except to bring the dose within range of the sensitivity of the method, can thus be avoided. As a general principle, it may be safer to use as an end-point any of the primary effects of TSH, rather than any of the second-order effects mediated through the thyroid. These objectives would not be difficult to attain, and a great service to this field would be done by the establishment of a standard to be used in conjunction with a modern method of assay.

Methods of Preparation of Thyrotropic Hormone

The methods used for preparation of thyrotropic hormone from either fresh or acetone-dried pituitary tissue will be reviewed here only in a general way, since White[2, 3] has discussed this subject in considerable detail. The methods can be conveniently arranged in three groups.

In methods of the first group, TSH is extracted from pituitary tissue and the extract prepared as a dry powder. Junkmann and Schoeller[1, 77] precipitated the initial alkaline pituitary extract with picric acid. After decomposition of the dye-protein complex, the active material was precipitated with acetone and dried. Rowlands and Parkes[95] precipitated TSH from alkaline extracts of the pituitary by means of alcohol. Such methods as these could be expected to lead to relatively little purification, since the reagents applied precipitate most proteins indiscriminately—the acid dyes by virtue of insoluble complexes with proteins and the organic solvents by virtue of protein insolubility, denaturation, or both.

More success attended the elimination of protein impurities of the extract before precipitation of the thyrotropic activity was carried out. Among methods of this second group, that of Loeser[104–112] can be cited. This author prepared alkaline extracts of pituitary tissue which were then treated with trichloracetic acid. Large amounts of protein were thus precipitated, but some thyrotropic activity remained in solution. The active substance was then precipitated from solution by excess acetone (90 per cent). Bergman and co-workers[113, 114] used flavianic acid to precipitate TSH from solution after the initial extract had been cleared of bulky protein impurities by isoelectric precipitation. The same general plan with a few modifications was used by others,[99, 115] who precipitated an alkaline extract with calcium phosphate to remove certain non-thyrotropic protein. The supernatant solution was then saturated with ammonium sulfate, thus precipitating thyrotropic hormone which, after dialysis, was dried by the addition of alcohol. Another modification of this general plan was worked out by Lambie and Trikojus,[116] who used sulfosalicylic acid to precipitate large amounts of protein impurities from the initial extract and then precipitated TSH with sodium tungstate. After decomposition of the tungstate, the active material was adsorbed by benzoic acid, eluted with acetone, and dried. Preparations made by this plan have yielded greater activities than those of the first, since non-thyrotropic proteins were first removed from the extract, after which the extract was prepared in dry form by one procedure or another.

In the third group, the methods involve the use of fractional precipitation of the initial extract after it has been cleared of as much non-thyrotropic impurity as possible. Fraenkel-Conrat and co-workers[117] achieved highly potent preparations by making 0.25 per cent acetic acid–1 per cent saline extracts of beef pituitaries. This is a good initial extract, for it is equivalent to the cleared extract achieved previously in two steps. The thyrotropic hormone was precipitated from this extract by the addition of one volume of acetone. The precipitate containing TSH was then fractionated between 0.3 and 0.6 saturated ammonium sulfate and again between 39 per cent and 90 per cent acetone. Finally, the Yale group of investigators[2, 118–121] have described a process for the isolation of the hormone in an electrophoretically pure form. The initial extracts were made with 2 per cent sodium chloride at pH 7.6 and protein impurities were removed by isoelectric precipitation at pH 4.1 and by precipitation from a 50 per cent acetone solution. The active material was then precipitated by the further addition of acetone to 75 per cent and the precipitate after solution in water was fractionated by lead acetate and trichloracetic acid. The acid solution was dialyzed and lyophilized. The resulting protein was found to be a pure protein by sedimentation and electrophoretic data. The molecular weight was calculated to be about 10,000 and the activity of the preparation was stated to be one chick unit per microgram.

Attempts to use physical methods of purification, such as ultracentrifuging crude extracts or partly purified preparations, were quite unsuccessful.[122, 123] The use of a wide variety of adsorbents[92] was only partly successful in certain instances and, consequently, further attempts utilizing this technique have been abandoned.

A comparison of these methods and the yields obtained are shown in TABLE 2.[77, 95, 113, 116–118] Some of the authors have not given sufficient data with which such tabulation could be arranged, and so a few personal guesses and averages were made. Of the six examples shown, five different methods of assay have been used. Since the units are different and not readily convertible, the only method of comparison of the final product obtained in each case is that of the degree of purification achieved over the starting source. This value can then be compared with similar values obtained by other workers, but the activity of the final product cannot be so compared. The methods of the first group give good yields but poor purification, since the general procedure amounts to no more than a drying of the initial extract of the pituitary. In the second group, in which impurities were first removed from the extract, after which the

Table 2

Comparison of Some Methods of Preparation of TSH from Beef Pituitary

Reference	*Start*			*Finish*			*Comparison*		
	Solids, kg.	*Units, $\times 10^3$*	*Units, per gm.*	*Solids, gm.*	*Units, $\times 10^3$*	*Units, per gm.*	*Yield solids, per cent*	*Yield units, per cent*	*Purification*
95	0.1	3 (?)	30	10	2.5	250	10	80	8X
77	1.0	375	375	20	400	20,000	2	100	50X
116	0.1	37.5 (?)	375	1.4	14	10,000	1.4	38	25X
113	0.66	33	50	7.8	31	4,000	1.1	90	80X
117	1.0	660	660	2.5	173	67,000	0.25	26	100X
118	0.2	625 (?)	3,100	0.5	500	1,000,000	0.25	80	320X

extract containing TSH was prepared in dry state, higher purification results. Fractional precipitation, characterizing the third group of methods, has yielded the highest purification. Ordinarily, great losses attend the use of fractional precipitation methods. Fraenkel-Conrat and co-workers have reported a loss of some 74 per cent of activity during manipulation, whereas Ciereszko[118] has apparently retained 80 per cent of the initial activity.

The efforts in this field have resulted in the isolation of an amorphous protein claimed to be pure TSH.[2] There are two types of criteria employed to assess the purity of a pituitary protein. The first type is physiological, *i.e.*, the hormone in question must not possess any physiological action attributed to other hormones of the pituitary as determined by appropriate biological tests. The second type is a physico-chemical proof, commonly applied to proteins in general. The first criterion has not been met. Fraenkel-Conrat and co-workers, who are singular in that they have reported in excellent detail the physiological properties of their product, stated that it was contaminated with prolactin to the extent of 0.025 per cent, adrenotropic hormone 3 per cent, growth hormone 1 per cent, FSH 0.4 per cent, and LH 10 per cent. Ciereszko[118] stated that 5 mg. of his amorphous protein did not give a reaction indicative of prolactin. He also stated that the product contained neither growth hormone nor gonadotropic hormone, but the doses used in performing these tests were not stated. If 1 mg. of a preparation gives no gonadotropic effect, it is not certain that 5 mg. might not give a positive reaction. In the absence of details as to dosage and of tests for other pituitary hormones, it would appear that the purity of the TSH isolated has not yet been proved by a sufficient number of the physiologic criteria. White[2] stated that a similar TSH protein prepared from sheep pituitary is twice as active as that prepared from beef (1 chick unit per 0.5 microgram), but that the product from sheep is contaminated with gonadotropic hormone. It is possible that the beef TSH protein could be pure and be simultaneously less than half as active as the same hormone prepared from sheep, a situation which would indicate, if true, that thyrotropin could exist as different proteins.

The chemical and physical criteria of purity are not within the scope of this discussion. Homogeneity in the ultracentrifuge or in the electrophoresis apparatus is part of the proof of purity. It is to be recalled that constant specific activity, crystallization, constant specific activity on repeated crystallization, and solubility data are other criteria to be applied. Data of this kind for TSH do not as yet exist.

No purified preparation of TSH comparable to those described is readily available for the thyroid physiologist, who must, by necessity, use relatively impure material. Since an impure TSH preparation is generally available (Antuitrin T, Parke, Davis & Company), and since most investigators in this country have been using it for the last ten years, it was thought worth while to explore the possibilities of improving its purity. Antuitrin T is available as a sterile solution in vials of 10 ml. each, 1 ml. containing 50 Junkmann-Schoeller units and 20 mg. of protein. The following method, which is simple enough to be carried out by technical aid, was worked out.[124]

The contents of a 10-ml. vial of thyrotropic extract are transferred to a 50-ml. centrifuge tube. Distilled water (10 ml.) is added to wash the vial and then is similarly transferred to the centrifuge tube. One volume (20 ml.) of 10 per cent trichloracetic acid is added to the centrifuge tube, the contents are mixed, and the tube is placed in a refrigerator for one hour at 5° C. A heavy, white precipitate is formed which is centrifuged off. The supernatant solution is stored and the precipitate is emulsified in 10 ml. of water. A few drops of 0.1 N NaOH are added until solution is complete. Trichloracetic acid (10 ml.) is again added and the tube is placed in the refrigerator for one hour. The heavy, white precipitate is centrifuged off and discarded and the combined supernatant fluid is adjusted to pH 7.0. Two volumes of saturated ammonium sulfate solution are added and the mixture is allowed to stand in the refrigerator overnight. The white precipitate is either filtered or centrifuged off, and the solution is discarded. The precipitate is dissolved in 10 ml. of water and 5 ml. of saturated ammonium sulfate solution is added. After standing from one to two hours in the refrigerator, a brownish precipitate is formed which is removed and discarded. To the supernatant solution is added 5 ml. of saturated ammonium sulfate solution and after standing overnight the precipitate is centrifuged off, dissolved in 5 ml. of water, and dialyzed against distilled water until salt-free. To the dialyzed solution is added M metaphosphoric acid to pH 3.0. The precipitate containing thyrotropic hormone which forms immediately is centrifuged off. The best preparations had an activity of 40 Junkmann-Schoeller units per mg. Eight milligrams of the preparation had no gonadotropic activity, which is present to an appreciable extent in Antuitrin T, owing to the separation and removal of FSH and LH during the procedure. When Ciereszko's method appeared, it was of interest to work up some Antuitrin T according to his procedure. Starting with the acetone precipitation step at pH 4.1, no increase in purity beyond 40 units/mg. was obtained. However, the failure to achieve a more potent product may have been due to differences in extraction and preparation of Antuitrin T.

TSH in Body Fluids

This aspect of the biochemistry of TSH is in a very confused state owing to the unsatisfactory nature of the bioassay and to the difficulties inherent in the protein nature of TSH. Since most current methods vary in sensitivity from 0.1 to 10 arbitrary units, they are not sufficiently delicate to detect minute amounts of hormone that may exist in body fluids, unless chemical methods are used to concentrate the material. For example, human blood serum may contain 1 arbitrary unit per 200 ml. of serum, an inference based on certain observations in the literature and some personal experience with human plasma. It would be impractical to withdraw the corresponding amount of blood from patients with thyroid disorders and, furthermore, this amount would suffice for only one test animal, provided the animal could tolerate it. A more realistic amount of blood to be withdrawn is 10 ml., which would contain, on the foregoing basis, a total of about 0.02 arbitrary unit, an amount of hormone which current methods cannot detect unless the serum is concentrated.

Similar difficulties apply to urine. If one considers only the positive results in the literature, one would estimate the TSH content to be about one unit per 200 ml. of urine. There is, of course, no problem in the amount of urine to be obtained, but it is not possible to inject such amounts unless the urine is concentrated by chemical or physical means. The common bond in this respect between blood and urine is that both may contain only traces of TSH. Otherwise, the chemical approach to the fractionation of blood and urine is different and will be described separately.

Tests on untreated blood serum (TABLE 3)[99, 124–146] in amounts up to 40 ml. per animal have yielded widely divergent results, and it cannot be stated that TSH has been unequivocally found in serum. The fractionation of serum for TSH has also yielded unsatisfactory results (TABLE 4).[147–151] Blood represents a concentrated solution of protein, and the problem is to remove as much non-TSH protein from the serum as possible while keeping TSH in solution, after which the protein fraction containing TSH can be precipitated. Remembering the adsorptive prop-

TABLE 3

SUMMARY OF SOME TESTS FOR TSH IN BLOOD SERUM (NO TREATMENT OF SERUM)

Reference	*Source of serum*	*Volume per test animal, ml.*	*Assay method*	*Results*
124–144	Rat, rabbit, dog	15	Guinea pig, histologic	+
	Guinea pig	15	Guinea pig, histologic	0
	Human (n., thy., myx.)	15	Guinea pig, histologic	+
	Human (obesity)	15	Guinea pig, histologic	0
	Human (acr., a.p. trauma)	15	Guinea pig, histologic	+
	Rat	40	Rabbit, histologic	+
	Human	40	Rabbit, histologic	+
145	Human (n.)	15	Guinea pig, histologic	0
	Human (thy.)	15	Guinea pig, histologic	+
	Human (acr.)	15	Guinea pig. histologic	0
	Dog (hyp.)	15	Guinea pig, histologic	+
	Dog (n., thyroidectomy)	15	Guinea pig, histologic	0
146	Human (thy.)	10	Hyp. rat, histologic	0
	Human (myx.)	10	Hyp. rat, histologic	+
99	Human (Simmonds')	?	Guinea pig, B.M.R.	Less than normal

Abbreviations: n.=normal; thy.=thyrotoxicosis; myx.=myxedema; acr.=acromegaly; a.p.=anterior pituitary; hyp.=hypophysectomized; Simmonds'=Simmonds' disease.

TABLE 4

SUMMARY OF SOME TESTS FOR TSH IN BLOOD SERUM (WITH CHEMICAL TREATMENT OF SERUM)

Reference	*Source*	*Treatment*	*Volume per test animal, ml.*	*Method of assay*	*Results*
147–148	Rabbit, human (n.)*	Acetone ppt.	10	Guinea pig, weight	0
	Human (thy.)	Acetone ppt.	10	Guinea pig, weight	0
	Human (myx.)	Acetone ppt.	10	Guinea pig, weight	0
	Rabbit (thyroidectomy)	Acetone ppt.	10	Guinea pig, weight	+
149–151	Human (n., thy., myx.)	Acetone ppt.	10	Guinea pig, histologic	+

* For explanation of abbreviations used previously see TABLE 3. Further abbreviations: ppt.=precipitate.

erties of bulky protein precipitates, the loss of TSH entailed in such procedures would be great and would tend to nullify the gains obtained by concentration. The reverse procedure of precipitating TSH selectively from the bulk of serum proteins seems impossible in view of the unselective nature of the precipitating reagents. For the fractionation of blood, advantage has been taken of the solubility of TSH in 40 to 50 per cent alcohol or acetone. The serum proteins insoluble in this concentration of reagent are removed, after which the proteins remaining in solution are precipitated by the further addition of reagent to 80–90 per cent. The results (shown in TABLE 4) obtained by use of this method again permit no definite conclusions regarding the presence or absence of TSH in serum.

A number of procedures for recovering TSH in blood, including the reported methods, have been studied.[152] For example, when 500 Junkmann-Schoeller units of pituitary TSH (Antuitrin T) were added to 500 ml. of whole blood, it was found that about 10 per cent of the TSH remained with the erythrocytes, probably by adsorption. About 20 per cent was found in the 40 per cent acetone precipitate, and 40 per cent of the activity seemed to have disappeared completely, leaving about 30 per cent actually recovered. The disappearance is puzzling but has been noted when other hormones were added to fresh serum and the mixture was immediately assayed. Other methods of extraction and concentration of TSH were also found to recover part of the activity. In any case, it seems certain that various methods are capable of recovering part of pituitary TSH added to blood, but the same methods applied to clinical material have not yielded indisputable evidence that TSH is actually present in blood.

The tests performed with urine are equally divergent. Amounts up to 50 ml. of untreated urine have been tested and it cannot be stated that TSH has been definitely demonstrated (TABLE 5).[124–144, 146, 153–160, 164, 165] The bulk of the work, however, has been done by concentrating human urine. Urine, unlike blood, is a very dilute solution of protein, and the problem is to remove the protein quantitatively from a large amount of solution. The losses of TSH thus entailed are equally large but are differ-

TABLE 5

SUMMARY OF SOME TESTS FOR TSH IN URINE (NO TREATMENT OF URINE)

Reference	*Source of urine*	*Volume per test animal, ml.*	*Assay method*	*Results*
124–144	Rat, dog, rabbit	15	Guinea pig, histologic	+
	Guinea pig	15	Guinea pig, histologic	0
	Human (n., thy., myx.)*	15	Guinea pig, histologic	+
	Human (pregnant)	40	Rabbit, histologic	0
153	Human (acr., Cushing's, n.)	15	Rabbit, histologic	+
154	Human (acr.)	15	Rabbit, histologic	+
155	Human (thy., p.o. exoph.)	30	Guinea pig, histologic	0
156	Human (n.)	16	Rabbit, histologic	+
146	Human (thy.)	50	Hyp. rat, histologic	0
	Human (myx.)	50	Hyp. rat, histologic	+
157–160	Human (thy.)	39	Guinea pig, histologic	0
164	Human	16	Guinea pig, histologic	0
165	Human (n.)	16	Rabbit, histologic	+(33%)
	Human (various disorders)	16	Rabbit, histologic	+(50%)

* For explanation of abbreviations used previously see TABLE 3. Further abbreviations: Cushing's= Cushing's syndrome; p.o. exoph.=postoperative exophthalmos.

ent from those attending the fractionation of serum, for they are due to incomplete precipitation, losses on glassware, and so forth. The methods used to recover TSH in urine have all revolved about the use of protein precipitants such as organic solvents, alkaloidal reagents, or adsorbents. Amounts of concentrate representing 50 ml. to 6 liters of urine have been tested for TSH (TABLE 6).[90, 97, 147, 148, 157–163] Again, one is not cer-

TABLE 6

SUMMARY OF SOME TESTS FOR TSH IN HUMAN URINE
(WITH CHEMICAL TREATMENT OF URINE)

Reference	*Source*	*Treatment*	*Volume per test animal, liters*	*Assay method*	*Results*
161	Pregnant	Tungstate ppt.	?	Guinea pig (?)	+
97	N., thy.,* thyroidectomy	Acetone ppt.	0.05	Guinea pig, cytologic	+
162	Myx.	?	?	?	0
90	Myx.	Acetone ppt.	0.2–0.4	Chick, cytologic	0 (1 case +)
	N., acr., Cushing's	Acetone ppt.	0.1	Chick, cytologic	0
163	N., cretin, myx.	Alcohol ppt.	0.2–0.5	Guinea pig, histologic	0
	Thy.	Alcohol ppt.	0.2–0.5	Guinea pig, histologic	0
	P.o. thy.	Alcohol ppt.	0.2–0.5	Guinea pig, histologic	+ (transient)
157–160	Thy.	Alcohol ppt.	2–6	Guinea pig, histologic, and B.M.R.	0
147–148	N.	Benzoic ads.	0.1–2.5	Guinea pig, histologic	0
	Thy.	Benzoic ads.	0.1–2.5	Guinea pig, histologic	12% of cases +
	Myx.	Benzoic ads.	0.1–2.5	Guinea pig, histologic	0

* For explanation of abbreviations used previously, see TABLE 3. Further abbreviations: ads.=adsorbate; p.o. thy.=postoperative thyroidectomy.

tain that this hormone has or has not been demonstrated as a constituent of urine.

The question always arises as to how completely such methods actually recover thyrotropic hormone, a question that can be answered at present only by adding pituitary TSH to urine and working up the artificial mixture. The recovery of TSH by all of the previously reported methods and by a few new ones has been studied.[152] Briefly, rich artificially prepared mixtures of urine and TSH, containing 1 unit per 30 ml. of urine, can be concentrated with recoveries of about 50 to 90 per cent. However, when poor artificially prepared mixtures containing 1 unit of TSH per 300 ml. of urine were used, only 20 to 50 per cent of the added TSH could be recovered. In general, the same impasse reached with blood has been attained in studies of urine—pituitary TSH can be recovered with varying success when it is added to urine, yet the application of the same methods to urine of patients has, on the whole, yielded conflicting results. There is also a contradiction in the literature, for positive reactions for TSH have been obtained with concentrates equivalent to 50 ml. of urine, and it is to be anticipated that ten times as much concentrate would induce a profoundly stimulating effect on the thyroid, a situation which has not often been reported.

It seems impossible to reconcile such conflicting data, but a few possible explanations can be considered and a few suggestions can be made. One factor may be that antithyrotropic substances of thyroid and extrathyroid origin may have been concentrated simultaneously with TSH, and thus the TSH effect of the concentrate would be nullified.[166] Another factor may be the unspecificity of the assay method. A careful analytical study has led to the conclusion that some impure pituitary TSH preparations induce histological signs of intense stimulation without increasing thyroid weight in the guinea pig, whereas this was not encountered with purified TSH.[103] Since concentrates from blood and urine are less pure than even the crudest pituitary extracts, it follows that these dichotomous effects constitute a danger in all studies utilizing histological or cytological methods of assay. A third consideration is that an assumption has been made that TSH in body fluids is the same substance as pituitary TSH, and that it can be recovered by methods which recover pituitary TSH when added to such fluids as a tracer. This is a dangerous assumption and one unsupported by any critical evidence.

In view of these considerations, it would be well to subject this field to a reinvestigation utilizing a bioassay delicate enough to detect from 0.01–0.001 arbitrary units of TSH. TSH action in untreated blood and urine having been demonstrated, the fractionation of these fluids can then proceed on the same basis as for the fractionation of any unknown substance for which a biological test exists. Only when this is done will it be possible to determine whether the thyrotropic hormone in body fluids resembles the hormone as obtained from the pituitary in its chemical or physical properties, or whether it circulates in the form of a new compound due to its metabolism or to its transport in blood. More important still is that, by simply increasing the dose of the purified concentrate, it will be possible to determine whether or not the concentrate possesses all of the physiological properties of pituitary TSH enumerated earlier. So far, it has been assumed that any one of the end-points (usually histological) constitutes *bona fide* evidence for the presence of TSH and, consequently, no one has tested the concentrate for all of the important actions of TSH. It is to be regretted that this precaution of conclusively demonstrating the physiological identity of urinary or blood concentrate with pituitary TSH seems to have been partly neglected.

Reactions of Thyrotropic Hormone

Most of the chemical reactions of TSH that have been reported are reactions of the hormone in impure state and will have to be repeated when enough pure material becomes available. The only basis used for evaluating these reactions is the loss or preservation of biological activity; certainly, chemical formulation in terms of composition or active groups is not possible.

In general, the reactions of TSH as a protein are not remarkable. Except for two divergent reports,[9, 11, 65] it is agreed that the biological activity is lost on boiling.[2] The divergent opinion may result from the fact that the effect of temperature on the biological activity of a protein depends, among other things, on the presence or absence of ions, the pH of the solution, and the presence or absence of impurities. TSH activity is destroyed by proteolytic enzymes such as pepsin, trypsin, and chymotrypsin, but not by papain and carboxypeptidase.[167] TSH is precipitated by several reagents which precipitate proteins, such as picric, picrolonic, tannic, phosphotungstic and flavianic acids, and by such salts as Reinecke's salt, mercuric chloride, gold chloride, silver nitrate, and uranium acetate. It is not precipitated by sulfosalicylic acid or by trichloracetic acid from purified solutions, but 60 to 90 per cent of the thyrotropic activity is carried with the bulky precipitate, presumably by adsorption, when these acids are used on impure pituitary extracts.[92] TSH is also readily adsorbed by a variety of materials, such as colloidal iron hydroxide, carbon, fuller's earth, permutit, ion exchangers, and benzoic acid.[1, 92, 168]

The solubility of TSH in salt solution such as ammonium sulfate and sodium sulfate leads one to the conclusion that its behavior is that of the so-called pseudoglobulin type of protein—being soluble in water and in dilute salt solutions but insoluble in more concentrated salt solutions (0.5 saturated ammonium sulfate solution). It is soluble in dilute solutions of organic solvents miscible with water such as 30 to 40 per cent alcohol or acetone, but becomes progressively insoluble in more concentrated solutions such as 60 to 80 per cent acetone. Advantage of this property has been taken in the purification of TSH. It will be recalled that TSH was precipitated from saline-acid extracts by adding acetone to 50 per cent by volume.[117] However, others[118] using essentially the same procedure found that TSH remained in the 50 per cent acetone solution. Like the effect of boiling, the solubility of TSH is dependent on temperature, pH, concentration of inorganic ions and other factors, and it is not surprising that even slight changes of conditions may profoundly influence the solubility of TSH in 50 per cent acetone.

TSH does not pass cellophane membranes. Because it is soluble in water throughout a rather wide range of hydrogen ion concentration, it was stated[99] that TSH has no isoelectric point. A direct determination of its mobility in an electric field has not been made, however, and it is to be recalled that this is the true basis for determination of an isoelectric point. TSH gives certain of the color reactions of proteins. Its elementary composition is not characteristic, *viz.*, 12 to 13 per cent nitrogen, 5 to 6 per cent hydrogen, 42 per cent carbon.[2] A content of 2.5 per cent of glucosamine has been reported.[117] Acetylation, benzoylation, and reduction by cysteine under certain conditions resulted in loss of thyrotropic activity.[117]

In addition to these reactions, the behavior of TSH to a variety of simple substances has been studied.[94, 152] As an example of the plan of these experiments, 6 mg. TSH (Antuitrin T) was added to a series of tubes containing 10 ml. of various oxidizing agents. The tubes were incubated for one hour at 37.5° C. and then diluted with distilled water to 45 ml. No attempt was made to buffer the system. One milliliter was injected subcutaneously daily for three days in a group of fifteen chicks, each chick thus receiving a total of 0.4 mg. or about 1 unit of TSH, a dosage of hormone which lies in a very sensitive portion of the dose-response curve. The results of the assay as determined by thyroid weight, mean cell height, and iodine concentration were expressed as percentages of the control standard.

The effect of certain oxidizing agents on thyrotropic potency is shown in TABLE 7. Permanganate and iodine induced complete inactivation of thyrotropic effect and were studied more extensively than the other

TABLE 7

EFFECT OF CERTAIN OXIDIZING AGENTS ON TSH*

Treatment	*Inactivation, per cent*
0	0
0.01 M $K_3Fe(CN)_6$	50
0.01 M $K_4Fe(CN)_6$	40
0.01 M $CrCl_3$	50
0.01 M $K_2Cr_2O_7$	35
0.01 M $KMnO_4$	100
0.01 M $Ba(MnO_4)_2$	100
0.01 M H_2O_2	20
0.01 M Na_2SeO_4	20
0.01 M I_2 in KI	100

* 0.4 mg. of TSH per chick.

agents. The effect of various concentrations of permanganate on TSH activity (TABLE 8) indicated that the degree of inactivation was proportional to the concentration of the reagent.[152] Simultaneous assay showed that permanganate abolished the net gonadotropic potency as well as the thyrotropic activity as determined by the weights of the testes and thyroid of the same test animal (TABLE 9). On the other hand, iodine seemed to have a more selective action on TSH than on gonadotropins (TABLE 10). Although not strictly comparable with the permanganate experiment, this experiment demonstrated that the TSH activity of Antuitrin T was reduced to the extent of 90 to 100 per cent, whereas the gonadotropic potency of the extract was not seriously affected.

TABLE 8

EFFECT OF $KMnO_4$ ON TSH ACTIVITY

TSH per chick, mg.	*Concentration of $KMnO_4$, molarity*	*Inactivation, per cent*
0	0	
0.8	0	0
0.8	0.1	100
0.8	0.01	100
0.8	0.001	76
0.8	0.0001	46
0.8	0.00001	0

TABLE 9

SIMULTANEOUS EFFECT OF $KMnO_4$ ON THYROTROPIC AND GONADOTROPIC POTENCY OF TSH EXTRACT

Extract per chick, mg.	*Treatment*	*Average thyroid weight, mg.*	*Average testes weight, mg.*
0	0	4.0	7.5
0.8	0	7.7	16.9
0.8	0.01 M $KMnO_4$	4.0	6.5
0	0.01 M $KMnO_4$	4.0	7.4

TABLE 10

SIMULTANEOUS EFFECT OF IODINE ON THYROTROPIC AND GONADOTROPIC ACTIVITY

Extract per chick, mg.	*Treatment*	*Average thyroid weight, mg.*	*Average testes weight, mg.*
0	0	3.19	9.20
0.8	0	6.60	16.40
0.8	Iodinated	3.25	12.00
8.0	0	9.60	26.30
8.0	Iodinated	4.90	26.50

The reaction of TSH and iodine merited further study in view of the well-known antithyrotropic activity of iodine on the thyroid and of its central role in the biosynthesis of the thyroid hormone. If a small amount of TSH extract (20 mg.) was mixed with 10 ml. of Lugol's solution and left at room temperature for half an hour to two hours, a brown precipitate appeared which was removed by centrifuging and washed with water. This precipitate, when properly diluted for assay, did not yield any thyrotropic effect; yet it represented most of the protein of the extract that was present in the mixture. Moreover, the activity was not present in the supernatant fluid or the washings. That the TSH was actually in the precipitate in an inactivated form will be made manifest later, since it was possible to treat this precipitate in many ways to recover the original potency. The reaction between iodine and the extract occurred in an acid medium (pH 5.0) and yielded a mixture of insoluble iodinated proteins, containing 10 per cent iodine. Part of the iodine (40 per cent) seemed tightly bound to the proteins, possibly absorbed, whereas 60 per cent was very loosely bound, perhaps adsorbed. It could be determined that the tightly bound iodine was not responsible for the loss of TSH activity, but that this was due to the loosely incorporated iodine.[94]

The presence of iodine in the inactivated TSH made it imperative to determine whether the inactivation was due merely to the biological action of iodine as such on the thyroid, or whether it represented a chemical alteration of the active protein of the extract. The first experiment consisted in adding variable amounts of iodine to a constant amount of TSH and comparing the potency of the mixture with the standard. It was found that the inactivation of TSH was dependent on the amount of iodine added. It was shown, furthermore, that no inactivation occurred unless the system contained free iodine. The minimal amount of iodine inducing maximal inactivation was calculated to be 100 micrograms of iodine per unit of TSH. This calculation was in fairly good agreement with the actual determination of free iodine in the iodinated precipitate (24 micrograms of iodine per unit of TSH). The difference of some 75 micrograms probably represented the excess free iodine in solution.

It was of interest to determine whether the inactivation of TSH was independent of the amount of iodinated TSH tested. In these experiments, variable amounts of TSH were added to an excess of Lugol's solution. The precipitates were removed and emulsified in water for assay. Whether 2 or 20 original units of TSH were given to each animal, the proportion of inactivation remained fairly constant, between 95 and 100 per cent.

That the inactivation of TSH was not due to the biological action of the iodine contained in the precipitate was determined by comparing mixtures of TSH with iodine as iodide, as molecular iodine in iodide, and as molecular iodine dissolved in alcohol, in various doses of from 10 to 10,000 micrograms. The results showed that iodide did not abolish the effect of TSH nor did it form insoluble precipitates of TSH, whereas free iodine in iodide or free iodine alone did. The effect of each form of iodine alone in varying amounts was of course nil, except to increase the thyroid iodine concentration slightly.

It was, therefore, apparent that the inactivation of TSH by iodine was not due to the biological action of iodine on the thyroid of the test animals. That it was not the result of any thyroxine forming in the reaction was indicated by mixing synthetic thyroxine in amounts exceeding the theoretical maximal yield of thyroxine that could have been produced by iodination of protein of the extract with TSH and assaying the mixture. Such a mixture resulted in only slight depression of TSH activity and could, therefore, not account for the complete loss observed. It was, furthermore, improbable that the loss of TSH activity could be attributed to an alteration of tissue absorption of the iodinated product, as, for example, some change induced by an irritating action of the free iodine. Furthermore, when the iodinated protein was dialyzed against large quantities of water, much of the excess free iodine was removed, and yet the dialyzed material remained completely inactive.[152]

The inactive iodinated material can be restored to activity by a variety of measures.[169] In these experiments, TSH was accurately added to a constant excess of Lugol's solution and the resulting precipitates were removed. After emulsification, the precipitates were triturated with various reducing agents in amounts just necessary to cause decoloration of the free iodine. In the first series studied, the reducing agents were 2-thiouracil, KSCN, $Na_2S_2O_3$, 2-aminothiazole, 6N propyl thiouracil, 5-amino-2-mercaptothiadiazole, ascorbic acid, and 3-phenylaminoethyl-2-mercaptothiazoline. When decoloration of iodine occurred, the precipitate immediately dissolved, yielding a water-clear solution much like the original Antuitrin T. The clear solution contained the original TSH activity that was added. Assuming that 5 per cent of the original activity remained in the inactivated precipitate (actually, none could be demonstrated), the thyrotropic potency of the iodinated product after reduction, as compared with the standard, was between 1,000 and 2,000 per cent greater than before reduction. A second series of substances consisting of benzyl thiouracil, cysteine, glutathione, tetramethylthiourea, 2-5 dithiothiadiazole, 2-thiobarbituric acid, and thiobarbital was also found to restore the activity of the iodinated complex. The extent of the reactivation, like that of the first series, was between 1,000 and 2,000 per cent as compared with the controls. In brief, these experiments showed that the restoration of activity was related to the reducing power of these agents, but not necessarily to their goitrogenic ability.

Since this reaction system contained three physiologically active reactants, it was essential to test them separately in all possible combinations. Thus, the same amount of reducing agents of the first series, when given alone, had no effect on the thyroid, nor did the same amount of iodine (200 micrograms) as contained in the iodinated precipitate when mixed with the reducing compounds induce any effect on the thyroid. The effect of iodine alone, of iodide mixed with the TSH, and of TSH alone has been described. From these control experiments, the conclusion was drawn that the restoration of TSH activity was due to an interaction of the iodinated complex and the reducing substances.

Since it might be thought that the reactivation resulted from some augmenting action of the reducing compounds on the assumed small portion of TSH escaping inactivation during iodine treatment, another type of experiment was done by mixing active TSH with these reducing substances in the same dosage. The results indicated that the biological potency of thyrotropic extract was enhanced over that of the control standard.[170] Furthermore, such an augmentation was present even after the reducing compounds had presumably been removed from the extract prior to bioassay by dialysis or after the hormonal material had presumably been removed from the agents by precipitation with ammonium sulfate. When the percentage augmentation was calculated per milligram of augmenting reagent, it was seen, in the first series, that goitrogens were far more effective than nongoitrogenic agents. Indeed, ascorbic acid induced a marked destruction of thyrotropic hormone, rather than augmentation.

The second series of agents was studied in a preliminary fashion for their augmenting ability.[152] Augmentation was induced by benzyl thiouracil, cysteine, tetramethylthiourea, 2-5 dithiothiadiazole, 2-thiobarbituric acid, and thiobarbital. However, cysteine did not affect TSH, and glutathione, like ascorbic acid, caused a decrease of TSH potency. Sufficient data were not available to determine whether in the second series there was still a correlation between the amount of augmentation and the goitrogenic potency of the various compounds.

The interactions of TSH, iodine, and goitrogens are of interest since all of these substances are in themselves physiologically active. Moreover, some of these effects have been duplicated *in vivo*.[171] It seems impossible, however, to attempt an explanation for them on chemical grounds. The TSH used in these experiments was quite impure, and it is not known that augmentation resulted from some alteration in the TSH molecule, or that it actually resulted from destruction of an inhibiting material present in Antuitrin T, or that some inactive material was converted. The effect of iodine may be thought of as an oxidation of proteins of the extract, including perhaps the protein representing TSH. The reactivation would seem to be due simply to reduction of the free iodine. The augmenting effect of reducing compounds might be related to their reducing potency, but this does not explain the reverse effects of ascorbic acid and glutathione. Possibly, the reducing potential is more important in this respect than the total reduction. Since the thyrotropic hormone represented less than 1 per cent of the total protein of the extract, it would be unwise to hazard a guess that these substances actually reacted with the TSH protein. Consequently, further exploration along these lines will be fruitless until such experiments can be repeated with pure thyrotropic protein, for only then will it be possible to obtain some definite clue as to the nature of the changes induced in TSH potency.

Aside from the physiological interest in such a study, it is of interest to learn what determines the biological activity of certain protein molecules. TSH has no prosthetic group, as far as can be determined, and so the biological activity may depend on the presence of certain chemical groups, or arrangement of these groups, or to some orientation of the groups to the molecule as a whole. Although many groups may be essential for physiological activity, it is possible that changes in any one group may lead to destruction of biological activity. It may be possible that some groups are more important than others in this respect. Consequently, acetylation of a protein, or changes in the S-S-bonds by thiol compounds, or substitution of iodine in the aromatic residues, may all seriously affect its biological activity, but it does not necessarily follow that one or another of these groups is responsible, *per se*, for the activity. To ascribe, as has been done, the physiological action of TSH to one or another group or, vaguely, to its "protein nature" is equally unsatisfying, but nothing better can be done until the chemistry of physiologically active proteins has been appreciably advanced.

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PHYSIOLOGICAL REACTIONS OF THE THYROID-STIMULATING HORMONE

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A VARIETY of thyroidal and extrathyroidal changes have been attributed to actions of the thyroid-stimulating hormone of the anterior pituitary. It is not the purpose of this paper to review the extensive literature concerning these varied effects observed in animals treated with pituitary extracts rich in thyroid-stimulating substances. Indeed, this discussion will be more or less limited to studies, done in our own laboratory, which we think tend to throw some light on the primary effects and mode of action of this hormone of the pituitary.

Some of the recent studies in which radioactive iodine has been used would indicate that the thyrotrophic hormone is related to the thyroid's metabolism of iodine. These studies merit consideration and discussion. Hertz and associates,[1] in some of the earlier studies with radioactive iodine, observed a parallel increase in the basal metabolic rate, thyroid hypertrophy, and collection of radioactive iodine in rabbits treated with thyroid-stimulating hormone. Leblond and Sue[2] observed that hypophysectomized rats concentrate much less of the administered tracer iodine in their thyroids than do intact controls. They also observed[3] that the thyroids of guinea pigs previously treated with thyroid-stimulating hormone trap more iodine in their thyroids than do untreated controls. On the basis of these observations, one might conclude that a primary action of the thyroid-stimulating hormone is to promote the collection and utilization of iodine. However, it might also be suggested that the increased collection of iodine by thyroids of animals previously treated with thyrotrophic hormone is independent of any direct action of the pituitary, but is related to an iodine want produced by a purging of the thyroid with TSH. This is suggested by the observations of Keating *et al.*,[4] who compared the anatomical changes with the loss and collection of radioactive iodine in cockerels treated with thyroid-stimulating hormone. In one study, they treated animals with one unit† of thyrotrophic hormone daily for 5 days. Groups of animals were sacrificed at the end of each 24-hour period, 4 hours after administering a tracer dose of radioactive iodine. The collection of radioactive iodine, thyroid weights and mean acinar cell heights were determined in each group of chicks. Results were calculated on the basis of per cent change over the controls. Though there were increases in the thyroid weights in the animals sacrificed at 24- and 48-hour intervals, a marked increase in weight was not manifest until after the third injection. The increase in mean cell height approached 200% in the first twenty-four-hour period, and 250% over the controls 48 hours after beginning treatment. There was no increase in the collection of radioactive iodine, however, until 48 hours after beginning treatment, at which time there was a hundred per cent increase in the collection of iodine. At the end of 96 hours there was a 500% increased collection of labeled iodine (see FIGURE 1).

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* Present address: Memorial Hospital, New York, N. Y.

† Junkmann-Schoeller units.

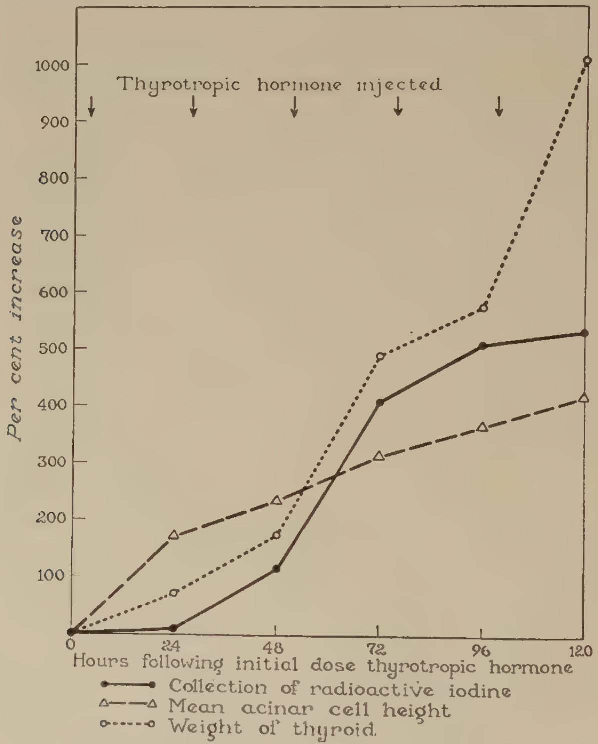

FIGURE 1. The effect of repeated doses of thyroid-stimulating hormone. Each group of animals was sacrificed 24 hours after the last injection of TSH and 4 hours after administering radioactive iodine.

In another group of experiments, these investigators followed the rate of iodine loss from the thyroids of newly hatched chicks. The chicks received a tracer dose of radioactive iodine before any food or water had been ingested. They then received one unit of thyrotrophic hormone daily for 3 days. One group was sacrificed every 24 hours up to 72 hours. The per cent of the previously administered radioactive iodine contained in thyroids at the time of killing was determined in each group. By comparing the values observed in the treated animals with those observed in chicks which received no TSH, the per cent loss of radio-iodine was determined. There was observed a 77% loss of the radioactive iodine 24 hours after the first injection of TSH. There was a loss of 93% and 96%

of the labeled iodine after 48- and 72-hour intervals (see FIGURE 2). Thus, it appears that the first changes to occur in TSH-treated chicks are a loss in thyroid iodine and an increase in the mean acinar cell height. The increased rate in collection of iodine did not occur until 24 hours later. In FIGURE 3, the per cent of iodine loss and the per cent of increased iodine collection are both plotted against time. From these curves, one might well obtain the impression that an increased iodine collection does not occur until after the thyroid has been purged of its iodine-containing hormone.

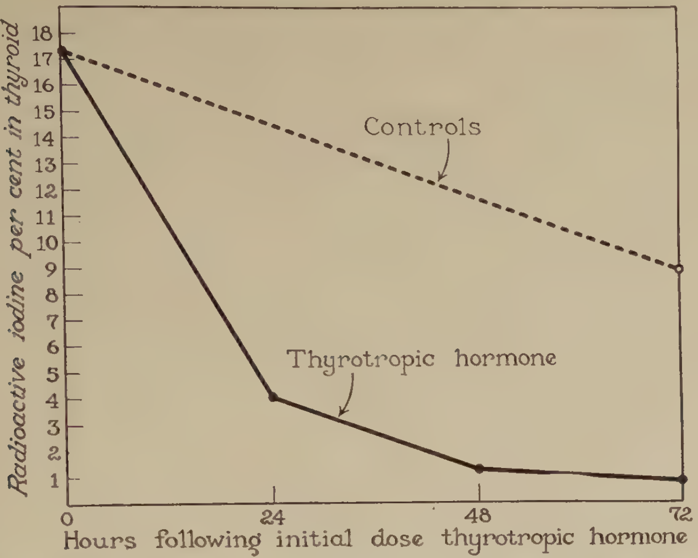

FIGURE 2. The effect of thyroid-stimulating hormone on the loss of iodine previously stored in the thyroid.

The observations of Morton, Perlman, Anderson and Chaikoff[5] would also indicate that the collection and utilization of iodine is not dependent upon an action of the thyrotrophic hormone. They observed that, though the thyroids of hypophysectomized rats collect less iodine than do those of the intact controls, they do collect a great deal more iodine than would be expected from simple diffusion. They observed that a large per cent of radio-iodine trapped by the thyroids of hypophysectomized rats is in the diiodotyrosine fraction. They found very little in the thyroxine fraction.

In another study done in our laboratory,[6] we have observed the rate of iodine loss and of changes in the mean acinar cell height after injecting one unit of thyrotrophic hormone. Cockerels five days of age, after having been on a diet of chick starter mash and tap water for 3 days, were given, by subcutaneous injection, one unit of thyrotrophic hormone. Ten treated and ten untreated controls were sacrificed every 6 hours for 48 hours.

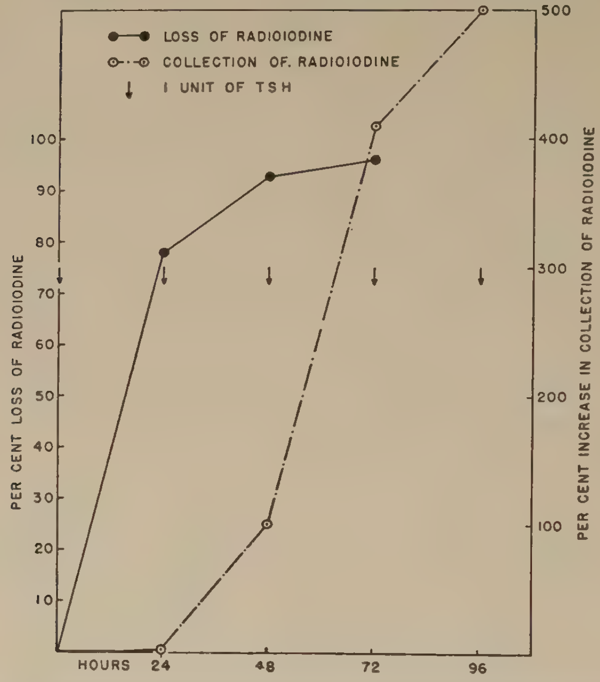

FIGURE 3. The per cent loss of radio-iodine and the per cent increase in collection of radio-iodine observed at varying time intervals after treatment with thyroid-stimulating hormone.

Four thyroid lobes were taken from each group of chicks and fixed in 10% neutral formalin. Paraffin sections 6 micra thick were prepared in the usual manner and stained with hematoxylin and eosin. Mean acinar cell heights were determined by the technique of Rawson and Slater.[7] The remaining 16 lobes of each group were equally divided and placed in vials containing 5 cc. of 2% KOH for digestion. Total thyroid iodines were done in duplicate on the digests by the method of Kendall[8] as modified by Astwood and Bissell.[9] A loss of thyroid iodine was observed in the treated groups as early as 6 hours after injecting the pituitary hormone. The maximum loss of iodine occurred 24 hours after injection of the TSH. After 30 hours, the direction of this curve was reversed. There was no demonstrable change in the mean cell height until 18 hours after injecting the TSH, 12 hours after the first significant change in thyroid iodine. The maximum change in mean cell height occurred at 30 hours, 6 hours after the peak in the iodine loss curve had been reached, following which there was a gradual return toward normal (FIGURES 4 and 5). Since it has been abundantly demonstrated that by far the major portion of iodine stored in the thyroid is in the thyroglobulin molecule, it is safe to say that the loss of iodine in these experimental animals is probably a loss of thyroid hormone. De Robertis[10] has reported that the colloid of thyroid follicles contains a proteolytic enzyme and that the pro-

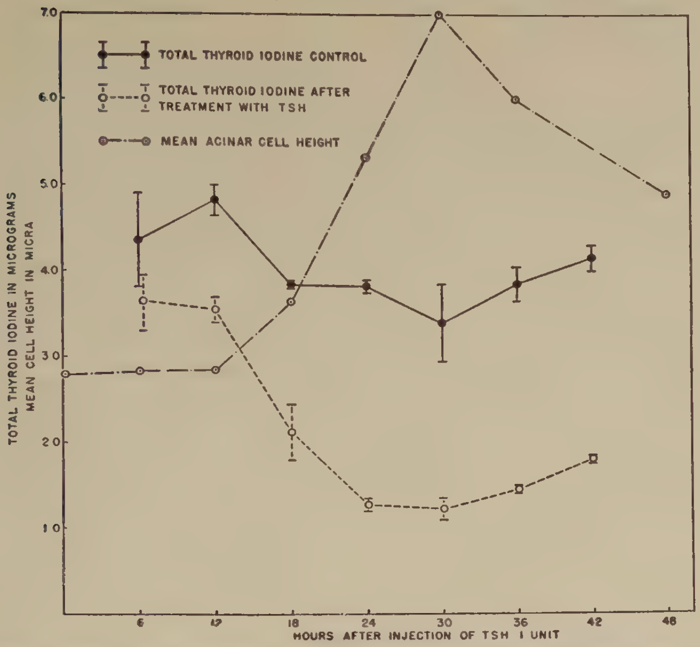

FIGURE 4. Thyroid iodine values and thyroid mean acinar cell-height measurements observed in cockerels at varying intervals following one injection of thyroid-stimulating hormone.

teolytic activity of droplets taken from thyroid follicles after the animals have been treated with TSH is increased. He has suggested the hypothesis that thyroglobulin is hydrolyzed in the lumen of the follicle by this proteolytic enzyme and that the smaller molecules resulting from this hydrolysis become available for transport through the follicular cells. If this concept is correct, it is quite likely that the loss of iodine observed by us in the thyrotrophic-hormone-treated cockerels is the result of an increase in proteolytic enzymes of the thyroid.

The effects that we have observed and the order in which they occur in the thyroids of animals treated with thyrotrophic hormone may be listed as follows: loss of thyroid iodine or thyroid hormone; increase in the thyroid acinar cell height; increase in thyroid weight; and finally an increase in capacity of the thyroid to concentrate iodine. It is an intriguing hypothesis that the primary effect of thyrotrophic hormone is to mobilize and release the thyroid hormone from the thyroid follicle and that the anatomical changes and increased avidity for iodine are secondary changes, not dependent on the pituitary. However, it is not proved.

Though the studies listed above would seem to indicate that at least one of the primary actions of thyrotrophic hormone is to liberate thyroid hormone from thyroid follicles, they do not throw any light on the mechanism of action of the thyroid-stimulating hormone. We have approached this by studying the effect of thyroid tissue on the thyroid-stimulating

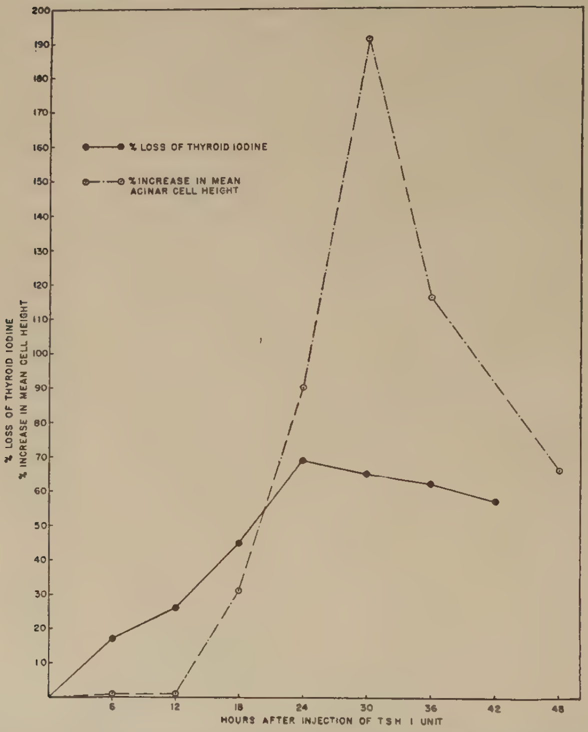

Figure 5. The per cent loss of thyroid iodine and the per cent increase in thyroid mean acinar cell height following one injection of thyroid-stimulating hormone.

effects of pituitary extracts. These studies were prompted by the clinical observations[11] that totally thyroidectomized patients excrete in their urines easily demonstrable amounts of active thyrotrophic hormone, whereas patients having untreated Graves' disease do not excrete active thyrotrophic hormone.

Tissue culture techniques were used in this study.[12] Roller bottles were coated with chicken plasma on which the tissue slices were clotted. The tissues were sliced and explanted in such bottles. The amount of thyroid tissue used in such experiments varied between 60 and 100 mgm. The tissues were then bathed in 15 cc. of Tyrode's solution containing thyroid-stimulating hormone in concentrations of $\frac{1}{4}$, $\frac{1}{2}$, and 1 unit per cc. (total of 3.5 to 15 units per bottle). The bottles were rotated 15 revolutions per hour in an incubator at 38°C. for a three-day period. At the end of the three-day period the medium was withdrawn and assayed for thyroid-stimulating effect.

Assays for the thyrotrophic activity were carried out on female guinea pigs weighing 180 to 220 grams or on cockerels. They were quantitated, in most instances, by determining the mean acinar cell heights of thyroids removed from the test animals as described by Rawson and Starr[11] and by Rawson and Salter.[7] In some instances when the assay was done on cockerels, the thyroid weights and thyroid iodines were also determined.

As one control, media containing the hormone in a concentration equivalent to $\frac{1}{4}$ of a unit per cc. were incubated in plasma-coated bottles containing no tissue for three-day periods. Other control studies included three-day incubations of other tissue explants with media containing the hormone in a concentration of $\frac{1}{4}$ unit per cc. The control tissues included rabbit adrenals, kidneys, livers, ovaries, pancreas, stomach mucosa, testes, thymus, and lymph nodes, and rat thymus and rat lymph nodes.

With these studies it has been consistently observed that there occurs a loss of thyroid-stimulating activity from the media exposed to explants of thyroid tissue (see FIGURE 6). With two exceptions, none of the control tissues have been observed to have any inactivating effect on the thyroid-stimulating hormone contained in the bathing media (see FIGURE 7). The exceptions were thymic and lymph nodal tissue. Both of these tissues have been observed to inactivate the hormone but to a lesser degree than does thyroid tissue. Under optimal conditions, we have observed that a normal rabbit thyroid will inactivate as much as 12 units of TSH. Lymph and thymic tissue will inactivate as much as 5 units of the hormone (see

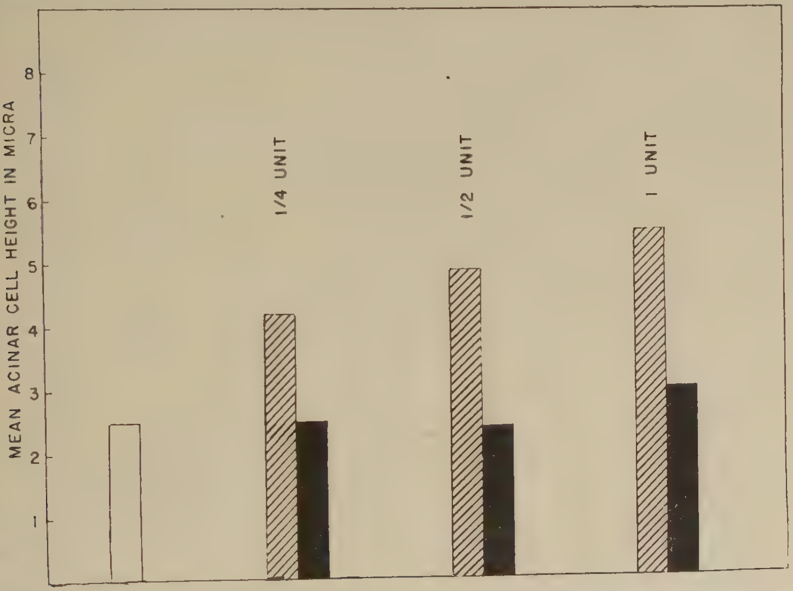

FIGURE 6. The mean acinar cell height of guinea pig thyroids after treatment with active and exposed (to explants of thyroid tissue) thyroid-stimulating hormone. The white column represents the thyroid mean acinar cell heights of untreated guinea pigs. The crossed-bar columns represent the thyroid mean acinar cell heights of animals treated with unexposed thyroid-stimulating hormone in the daily doses indicated. The solid black columns represent the thyroid mean acinar cell heights of guinea pigs treated with hormone following its exposure to explants of thyroid tissue.

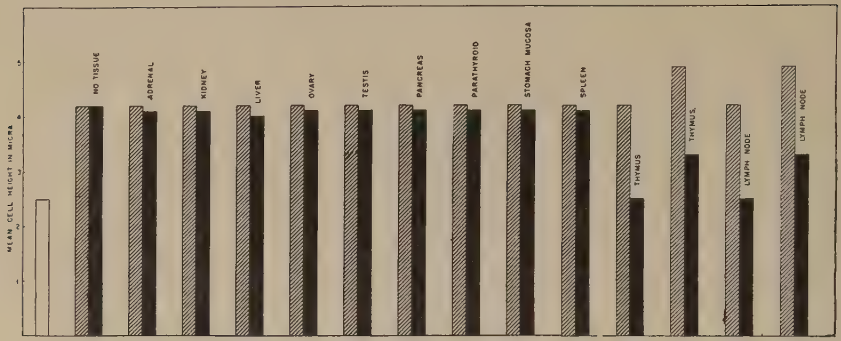

FIGURE 7. The mean acinar cell heights of guinea pig thyroids following treatment with $\frac{1}{4}$ unit daily of unexposed thyroid-stimulating hormone illustrated by crossed-bar columns, and with the same amount of hormone following its exposure to explants of the tissues indicated, illustrated by the solid black columns. Thyroid-stimulating hormone in concentrations of both $\frac{1}{4}$ and $\frac{1}{2}$ units per cc. was exposed to explants of thymus and lymph nodes. The mean cell heights of untreated control guinea pig thyroids is indicated by the white column.

FIGURE 8). During the past 18 months, we have observed more variability in the capacity of rabbit thyroids to inactivate the hormone. On some occasions, one rabbit thyroid has been observed to inactivate as little as 3 to 4 units of the hormone. We suspect that this is probably due to the fact that we have been competing with the human consumers of rabbits for our animals. It has been suggested by some that this is due to changes in the nutrition of rabbits that we are able to purchase.

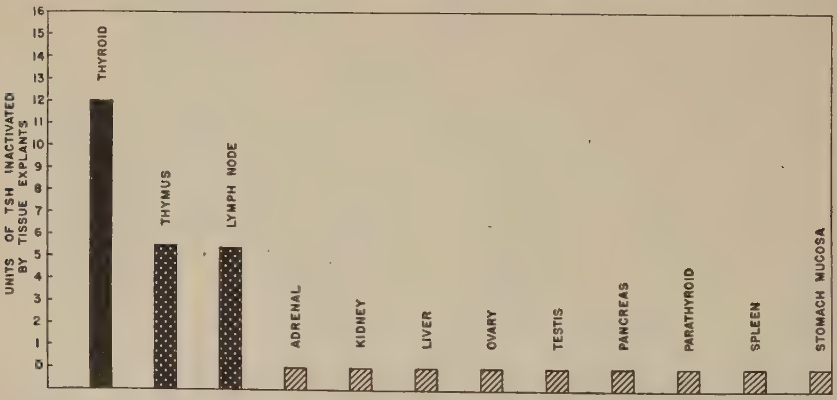

FIGURE 8. The amount of thyroid-stimulating hormone inactivated under optimal condition by the tissues indicated.

With Dr. Brown M. Dobyns,[13] we have observed that incubating pituitary extracts with thyroid tissue results in a loss of the exophthalmos-producing factor. Dobyns[14] has made daily measurements on the intercorneal distance of 300 to 400 gram thyroidectomized guinea pigs before and during treatment with pituitary extracts rich in thyrotrophic hormone. He has observed that thyroidectomy alone will cause a measurable increase in the intercorneal distance. However, the administration of certain extracts made from beef pituitaries (Antuitrin T, Parke Davis) produced an acute and marked increase in the intercorneal distances of thyroidectomized guinea pigs. Administration of purified extracts made from swine pituitaries resulted in much less exophthalmos. Using the same preparation that Dobyns found to be rich in the exophthalmos-producing factors we have explored the possibility of inactivating this factor of the pituitary by exposure to rabbit thyroid tissue.

In this study, we have pooled the media after exposure to thyroid tissue, concentrated it by precipitating the hormone with acetone, and then redissolved the hormone in water. Thyroidectomized guinea pigs were treated with an original equivalent of 25 units of exposed hormone per day for 7 days. There were two control groups, (*1*) untreated thyroidectomized guinea pigs, and (*2*) thyroidectomized guinea pigs treated with unexposed active thyroid-stimulating hormone which had been diluted to the same volume in Tyrode's solution, precipitated with acetone, and redissolved in an aqueous solution. Daily measurements of the intercorneal distance of each guinea pig were made by Dobyns's technique. The results were then plotted graphically (see FIGURE 9). At the end of 7 days, the animals which received unexposed thyrotrophic hormone had developed the classic picture of experimental exophthalamus. There occurred no exophthalamus in either the untreated guinea pigs or in the animals which received thyrotrophic hormone after it had been incubated with thyroid tissue. These observations may suggest that the exophthalamus-producing factor of the pituitary is closely related to TSH if not the same molecule.

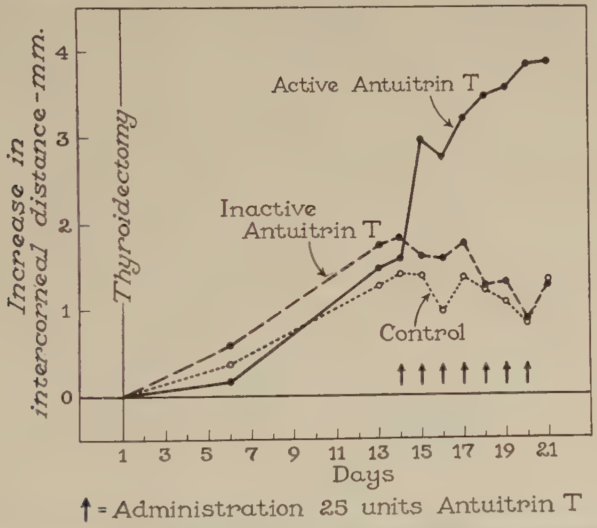

FIGURE 9. The effects of active and inactivated thyroid-stimulating hormone preparations on the intercorneal distances of thyroidectomized guinea pigs.

In another study,[15] we have used tissue-culture techniques to determine the effect of normal and pathologic thyroid tissue removed from humans on the activity of thyroid-stimulating hormone. The same methods were used as those described above. In each experiment, approximately 150 to 200 mgm. of thyroid tissue were explanted. The thyroids of patients having Graves' disease were removed after obtaining a maximum response to treatment with iodine. The pretreatment basal metabolic rate levels of these patients ranged between +25 and +65. Nontoxic nodular goiters were also obtained from routine operations for such goiters. The basal metabolic rates of these patients were all below —10. Biopsy samples of normal human thyroid tissue were taken by Dr. Oliver Cope at operations for parathyroid tumors. It was observed that explants of nodular thyroids which, on histologic examination, showed colloid nodules, inactivated none of the exposed thyrotrophic hormone. Similar explants of normal human thyroid tissue were found to inactivate between 3 and 4 units of the hormone. The tissue explants taken from patients suffering with Graves' disease were found to inactivate between 6 and 7.5 units of TSH (see FIGURE 10). It might be suggested that this increased reactivity between the thyroid of Graves' disease and the thy-

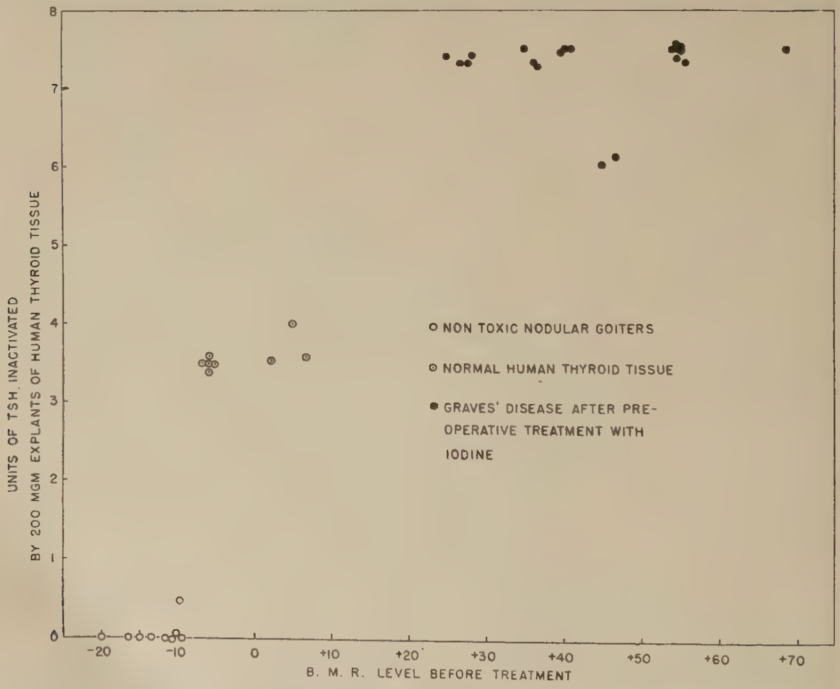

FIGURE 10. The quantity of thyroid-stimulating hormone inactivated by exposure to explants of normal and pathologic human thyroid tissue.

roid-stimulating hormone was due to an increased cell mass in these goiters. However, with few exceptions these glands were found to be involuted and two of them showed hyperinvolution. We have suggested that this reaction might explain our failure to demonstrate active thyroid-stimulating substances in the urine of untreated thyrotoxic patients. Indeed, we have suggested the hypothesis that this increased reactivity between the thyroid-stimulating hormone and thyroid tissue of patients with Graves' disease may be the key to an important etiologic factor in this malady.

The nature of this reaction between thyroid tissue and the thyrotrophic hormone is not known. It has been suggested that it is non-specific and possibly proteolytic in nature. Those who believe that the exophthalmos-producing factor of the pituitary is separate from the thyrotrophic factor may cite the observations that the exophthalamus-producing factor of pituitary extracts is lost following exposure to thyroid tissue in support of the thesis that the reactions we have observed are non-specific. However, by observing changes in the weight of testes taken from cockerels treated with pituitary extracts after exposure to thyroid tissue, we have demonstrated that inactivation of the thyroid-stimulating hormone by thyroid tissue is not paralleled by any loss of gonadotrophic activity. We can say then that this reaction is not a non-specific one affecting all pituitary hormones. It has been suggested that the inactivation of thyrotrophic hormone by thymic and lymph nodal tissue speaks against any specificity of this reaction. On the other hand, however, it has long been known that clinical Graves' disease is associated with a lymphoid hyperplasia and an enlargement of the thymus. Thus, that these tissues inactivate the thyrotrophic hormone as does the thyroid, may increase the significance of these observations that we have made.

We favor the hypothesis that the inactivation of thyrotrophic hormone by thyroid tissue is an oxidative phenomenon in which the thyroid-stimulating hormone contributes an integral part of its molecule to the metabolism of thyroid tissue. This theory is supported by the observation[16] that thyroid-stimulating hormone inactivated by exposure to thyroid tissue can be reactivated when treated with certain reducing agents which also are goitrogenic. In this study, explants of rabbit thyroid have been bathed in Tyrode's solution containing thyrotrophic hormone in a concentration of $\frac{1}{4}$ unit per cc. for 3-day periods. The media were pooled and assayed. After the hormone had been demonstrated to be inactive, the pooled media were treated with reducing agents (in concentrations too small to produce goiters). We have observed that hormone demonstrated to be inactive, after incubation with thiouracil, recovers a major part of its original hormonal activity, *i.e.*, capacity to increase thyroid weight and to decrease thyroid iodine in the cockerel (see FIGURE 11). We have also observed that incubation of inactivated thyrotrophic hormone with 5 aminothiodiazole, 2 thiol, and 3 (phenylaminomethyl) thiazoline

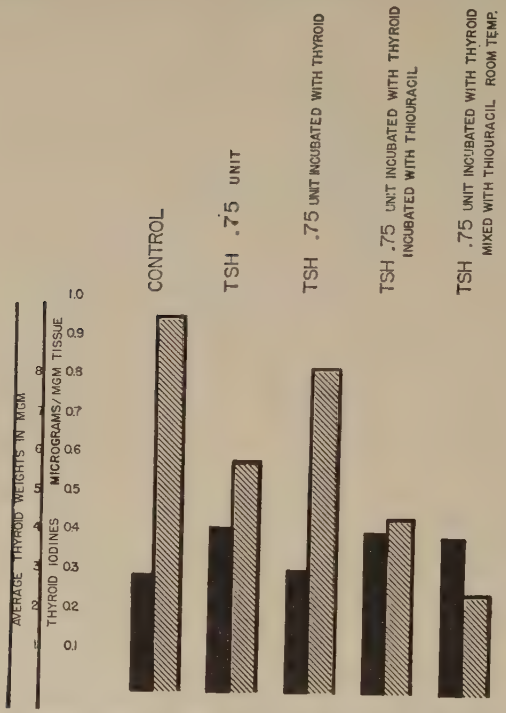

FIGURE 11. The effects of active thyroid-stimulating hormone following its exposure to rabbit thyroid tissue before and after treatment with thiouracil on the thyroid weights and iodine content in cockerels. The black columns illustrate thyroid weights, the crossed-bar columns thyroid iodines.

2 thione results in a recovery of the thyroid-stimulating action of the hormone mixtures. The latter two reducing agents in the intact rat have a goitrogenic activity comparable to that of thiouracil.

Further support for the thesis that the inactivation of the thyroid-stimulating hormone is an oxidative one is found in the observation reported by Dr. Albert[17] that this hormone loses its activity upon exposure to an oxidizing agent such as elemental iodine and that this inactivation of the hormone can be reversed when the inactivated hormone is exposed to a variety of reducing agents, most of which are goitrogenic.

These observations may indicate that the goitrogenic action of thiouracil and related agents is not wholly on the basis of thyroid inhibition. Indeed, we have observed that the administration of a standard amount of thyroid-stimulating hormone to chicks receiving thiouracil in the diet resulted in a marked augmentation of thyrotrophic potency when compared with controls receiving no goitrogen. Administration of thyrotrophic hormone after treatment with elemental iodine, which was inactive when given to controls, resulted in appreciable thyrotrophic effects in chicks receiving thiouracil (see TABLE 1).

In another group of experiments, we have studied the effect of iodide

TABLE 1

EFFECT OF ACTIVE AND INACTIVE TSH ON THE THYROIDS OF CHICKS ON NORMAL AND THIOURACIL DIET

*Agents per chick**	*Normal diet*		*Thiouracil diet*	
	Thyroid weight *mg.*	*Thyroid I* *µg. per mg.*	*Thyroid weight* *mg.*	*Thyroid I* *µg. per mg.*
None	4.00	0.78	4.28	1.68
I–TSH†	3.70	0.76	5.80	0.08
TSH	6.50	0.07	9.14	0.00

* 100 Chicks total, 10 animals per subgroup. Average body weights varied from 45–50 gm. at end of experiment.
† I–TSH and TSH are abbreviations, respectively, for iodinated thyrotrophic extract, and active (untreated) thyrotrophic extract.

on the *in vitro* reaction between thyroid tissue and the thyrotrophic hormone. Explants of rabbit thyroid tissue were bathed in 15 cc. of Tyrode's solution containing 7½ units of thyrotrophic hormone (½ unit per cc.), and iodine as sodium iodide in a concentration of 100 micrograms per cent. At the same time, similar explants of thyroid tissue were bathed in media containing the same amount of TSH without iodide. The degree of inactivation of the TSH was quantitated by microhistometric methods applied to thyroids of chicks after treatment with the various media. Complete inactivation of the hormone resulted from exposure to slices of thyroid tissue without iodide. However, when TSH was exposed in the presence of iodide, very little inactivation of the hormone occurred. It was calculated that, at most, less than one of the 7½ units was inactivated (see FIGURE 12). On the basis of these observations, we have suggested that the therapeutic effect of iodine, when administered to patients with Graves' disease, is due to a blocking effect of iodide to the reaction between the thyrotrophic hormone of the pituitary and the thyroid cell.

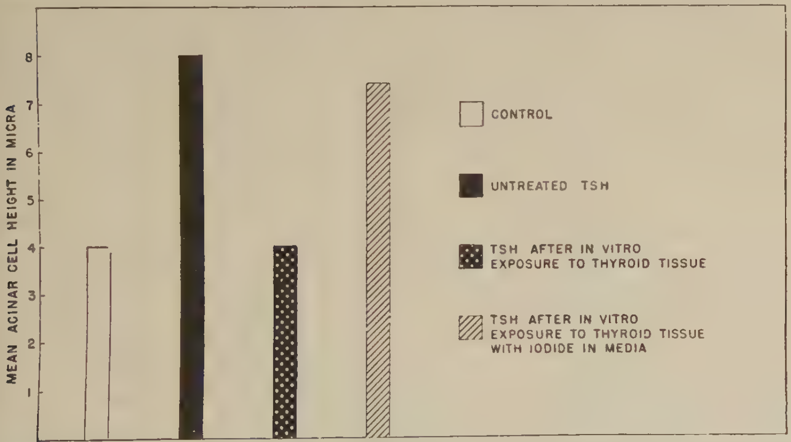

FIGURE 12. The effect of iodide in the bathing medium on the inactivation of thyroid-stimulating hormone by thyroid tissue.

This theory is supported by the observation that administering iodine inhibits the histologic changes produced in hypophysectomized rats treated with thyroid-stimulating hormone. Male rats of the Sprague Dawley strain weighing 50 to 60 gm. were hypophysectomized. One month after operation they were treated with 8 units daily of TSH for 4 days. Iodine was administered to two groups of animals as sodium iodide in daily doses of 500 and 1000 micrograms during the period of treatment with thyrotrophic hormone. The animals were sacrificed on the 5th day. Thyroids of the rats proved to be hypophysectomized were fixed in (10% neutral) formalin. Paraffin sections were made and the mean acinar cell height of each thyroid was determined. Thyroid mean acinar cell heights of the untreated control rats averaged 3.8 micra; of rats treated with 8 units of TSH daily, 10.1 micra; of rats treated with TSH 8 units and NaI 1000 micrograms daily, 5.2 micra; of rats treated with TSH 8 units daily and NaI 500 micrograms daily, 5.4 micra (see FIGURE 13). Thus, it can be said that the reaction between thyrotrophic hormone and thyroid tissue can be prevented by iodides not only *in vitro* but also in the hypophysectomized rat. We have not explored the possibility of iodine inhibiting the pituitary's elaboration and secretion of the thyroid-stimulating hormone. However, we feel justified in advancing the hypothesis that at least part of the therapeutic action of iodine in Graves' disease is an inhibition to the reaction between thyroid tissue and the thyrotrophic hormone.

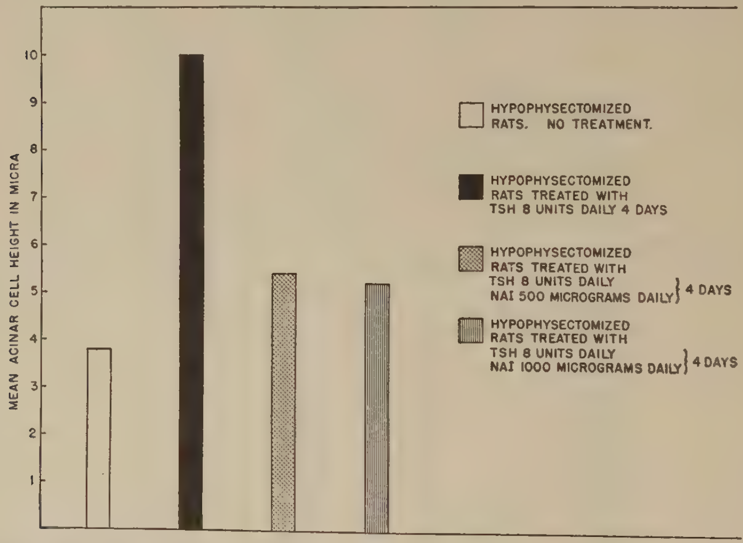

FIGURE 13. The effect of thyroid-stimulating hormone on the mean acinar cell heights of hypophysectomized rats when given with sodium iodide.

In a similar study[20] done on hypophysectomized rats, it has been demonstrated that the effect of thyroid-stimulating hormone on the thyroid cell can also be inhibited by administering thyroxine. It has been demonstrated by several investigators that administering an excess of exogenous thyroid substance puts the recipient animal's own thyroid in a resting state.[21] It has also been demonstrated that the simultaneous administration of thyroid hormone with an active thyrotrophic hormone of the pituitary prevents, in part, the usual thyroid-stimulating effect of the pituitary extract.[22] It has been postulated that this effect of thyroid hormone was the result of suppressing the animal's own pituitary by the administered thyroid substance.[23] The present study was undertaken to determine whether the inhibitory action of thyroid hormone could be, in part, due to an effect on the thyroid cell. Male rats of the Sprague Dawley strain weighing between 70 and 90 grams were hypophysectomized, and the capacity of such animals to respond to thyroid-stimulating hormone was compared to that of similar animals receiving thyroxine. The thyroxine was administered in daily doses of 20 micrograms for 10 days. The thyrotrophic hormone was administered in daily doses of 4 Junkmann Schoeller units the last 4 days of each experiment. The responses of these animals to the thyroid-stimulating hormone were quantitated by determining the thyroid mean acinar cell heights and by measuring the thyroid's avidity for radioactive iodine. The mean acinar cell heights of untreated controls averaged 2.6 micra; of rats treated with TSH alone, 6.5 micra; of rats treated with thyroxine plus TSH, 4.8 micra; the per cent collection of the administered radioactive iodine by the unoperated untreated controls averaged 6.9%, the operated untreated controls, 0.4%, the hypophysectomized thyroxine-treated rats, 0.4%, rats treated with TSH alone, 7.8%, rats treated with thyroxine and TSH, 2.7%. Thus we have evidence that thyroxine interferes with the action of TSH on the thyroid cell. We have not studied the effect of thyroxine on the *in vitro* reaction between the thyroid cell and the thyroid-stimulating hormone.

Summary

1. We have observed the following effects, in the order given, in thyroids of cockerels treated with thyroid-stimulating hormone: loss of thyroid iodine, increase in thyroid-acinar-cell increase in thyroid weight, and finally an increased capacity to concentrate radioactive iodine.

2. With *in vitro* techniques we have observed that the exposure of thyroid-stimulating hormone to explants of rabbit thyroid tissue results in a loss of the hormone's activity. Exposure of the hormone to explants of thymus and lymph nodes taken from rabbits and rats also resulted in loss of activity, but to a lesser degree than that caused by thyroid tissue. Incubation of the hormone with explants of adrenals, kidneys, livers, ovaries, pancreas, stomach mucosa, and testes from rabbits resulted in no loss of the hormone's thyroid-stimulating effect. Loss of the thyroid-stimulating effect upon exposure to thyroid tissue was not paralleled by any loss of gonadotrophic activity of the pituitary extract used in this study.

Explants of normal thyroid tissue weighing 150 mgm. were observed to inactivate between three and four units of the thyrotrophic hormone. Similar explants taken from nontoxic colloid goiters failed to inactivate the hormone. Explants of the same weight taken from the involuted thyroids of patients with Graves' disease after treatment with iodine were observed to inactivate between 6 and 7.5 units of the hormone.

3. Hormone inactivated by exposure to thyroid tissue has been reactivated by certain reducing agents which are goitrogenic.

4. Iodide has been observed to inhibit the inactivation of the thyroid-stimulating hormone by slices of thyroid tissue. It has also been observed to inhibit the action of TSH on the thyroid of hypophysectomized rats.

5. The administration of thyroxine has also been observed to inhibit the action of the thyroid-stimulating hormone on the thyroid of hypophysectomized rats.

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